AN ATTEMPT TO PRODUCE HEMOCHROMATOSIS EXPERIMENTALLY AND THE EFFECT OF HIGH LEVELS OF COPPER AND CHOLINE IN THE DIET [1]

NATHAN KAUFMAN, J. A. CARTAYA, PHILIP L. WHITE,[2] D. MARK HEGSTED AND THOMAS D. KINNEY

Departments of Pathology of the Cleveland City Hospital and of Western Reserve University School of Medicine, Cleveland, Ohio, and Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

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This study was initiated in order to determine if lesions resembling hemochromatosis could be produced experimentally by a combination of circumstances favorable for both excessive iron absorption and the development of cirrhosis. A diet low in protein and choline and containing excessive iron or copper, or both, was selected. Rats were used as experimental animals.

Briefly, the reasoning which led to the selection of this diet was as follows: excessive iron must be absorbed in hemochromatosis, since it has been repeatedly shown that iron is not excreted in appreciable amounts. Excessive iron in the diet has been shown to increase iron absorption (Hegsted et al., '49). A low protein, low choline diet is known to produce cirrhosis (Daft et al., '41). Liver disease of this severity might affect iron absorption indirectly by interference with protein metabolism. Finally, excessive copper was fed be-

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[2] U. S. Public Health Service Predoctorate Research Fellow, National Heart Institute.

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cause of the report of Mallory, Parker and Nye ('21) that chronic copper poisoning in animals produced a condition which approximated hemochromatosis. No consideration was given to the diet in these early studies.

It is realized that no evidence is at hand to indicate that hemochromatosis, as seen in this country, is associated with malnutrition. This was not considered sufficient reason to discourage the work, since excessive iron absorption and the development of hemosiderosis are apparently related to faulty nutrition in South African natives (Gillman and Gillman, '45).

There is some evidence that iron and copper may be interrelated in other ways than the well-known fact that both are required for hemoglobin formation. Gillman, Mandelstam and Gillman ('45) found that the copper level was generally high in a group of human livers which contained increased amounts of iron. A similar tendency in iron-heavy livers has been reported by Bence ('34). Sheldon ('35) indicated that liver copper was increased in hemochromatosis.

None of the animals in this study developed a lesion in the liver closely resembling that appearing in the liver in cases of hemochromatosis in man. We were therefore unsuccessful in the original purpose of the experiments. However, a heretofore unsuspected interrelation between copper and choline appeared, which was considered worthy of presentation.

EXPERIMENTAL

The low protein, low choline diet, called the basal diet, was of the purified type and had the following percentage composition: sucrose or glucose 77, vitamin-free casein 8, salt mixture 4 (Hegsted et al., '41), corn oil 10 and cod liver oil 1. Crystalline vitamins were added to supply the following amounts per 100 gm of diet: thiamine chloride 400 μg, pyridoxine hydrochloride 400 μg, riboflavin 800 μg, calcium pantothenate 1.5 mg and nicotinic acid 2.5 mg. The quantities of ferric citrate powder (U.S.P. VIII), $CuSO_4 \cdot 5H_2O$, choline chloride and casein that were used as supplements are shown

in the accompanying tables. When additional casein was added, it replaced an equivalent amount of sugar. All diets were fed ad libitum.

Adult male ablino rats were used throughout. The animals were housed in individual cages in experiments I and II and in groups of three or 4 animals per cage in the other experiments. Animals were killed at intervals in experiments I and II, starting after 10 weeks in experiment I (when the first animal in group 4 died), and after 6 weeks in experiment II. This has made the analysis of the data in these experiments somewhat difficult, but since animals from each group were killed at approximately the same time, we have ignored this variable in analyzing the data, although this may account for the greater variability in these experiments. All animals in experiments III, IV and V were killed at the same time.

At autopsy, portions of the various organs were removed and placed in Zenker's solution and formalin for histological examination. The livers were stained with hematoxylin and eosin, as well as for iron, using a modification of Perl's stain. Stains for fat and reticulum were done. In experiments I and II a portion of the liver was digested with alcoholic KOH and the fatty acids separated and titrated with standard alkali. In experiment III the total ether extract of the dried livers was determined. In the first two experiments a portion of the dried liver was ashed at 400°C. Iron and copper were determined, using orthophenanthroline and the dithiazone procedure, respectively. In the latter experiments the wet ashing procedure of Jones ('48) for the determination of iron and copper was used, with more consistent results.

Variance analysis was used wherever possible to determine the statistical significance of the results obtained and whether the various substances tested — i.e., copper, iron and choline — showed a significant interaction. A significant interaction means that the effect of the combined supplements was statistically different from that expected on the basis of the results obtained with the same substance used alone.

RESULTS

As stated earlier, experiments I and II were the original studies which led to a more intensive investigation. An inspection of the weight gains presented in table 1 and the results of the statistical analysis in table 2 indicated several interesting relationships:

(a) Over a three- to 4-month period neither iron nor copper alone at the levels fed was detrimental, although the combination of iron and copper was definitely toxic.

(b) The same combination of iron and copper was not toxic when casein and choline were also included in the diet.

(c) The addition of choline without casein did not exert a protective effect (experiment II) but actually increased the toxicity. Group 2 gained 46 gm less than group 1, whereas group 4 gained 83 gm less than group 3. This interaction approached statistical significance (table 2).

The chemical and histological studies on the livers of the animals from experiments I and II suggest that choline supplementation increased the liver copper and possibly the liver iron, while casein prevented this accumulation. Liver fat was usually lower in the groups receiving copper or copper and iron. These results were not significant statistically because of the great variation encountered in the individual animals.

The results of the more extensive experiments on copper and choline (experiment III, table 1, and experiment V, table 3) leave no doubt of the significance of the copper-choline interrelationship. In experiment III, copper produced considerable weight loss over the 8-month period and choline increased the rate of gain, as would be expected with this choline-deficient diet. The combined supplements, however, were much more toxic than copper alone. Each of these effects, including the interaction of copper and choline, was highly significant. The results of experiment V (tables 3 and 4) give additional evidence of the harmful effect of choline in the presence of copper and also show that it is a quantitative effect. The weight loss was dependent upon the level of

TABLE 1

The effect of various supplements on the weight and liver composition of adult rats receiving a low-choline, low-protein diet

EXP. NO.	NO. OF RATS AND DURATION OF EXP.	GROUP NO.	DIETARY SUPPLEMENTS	AVE. GAIN	LIVER ANALYSES				HISTOLOGICAL [2] FAT AVE. SCORE
					Copper		Iron	Fat [1]	
					Ave.	Range			
				gm	*μg/gm*	*μg/gm*	*mg/100 gm*	%	
I	5/group	1	None	3	51	36–90		7.4	3.7
	3–4 months	2	0.1% $CuSO_4 \cdot 5H_2O$	33	479	60–1,060		5.2	3.3
		3	2.2% Fe citrate	34	96	52–138		9.0	3.8
		4	0.1% $CuSO_4 \cdot 5H_2O$ + 2.2% Fe citrate	−46	522	154–900		11.0	4.0
		5	10% Casein + 0.3% choline	117	79	44–144		1.6	0
		6	10% Casein, 0.3% choline, 0.1% $CuSO_4 \cdot 5H_2O$ + 2.2% Fe citrate	111	82	44–160		1.6	0
II	7/group	1	None	19	52	14–60	32	18.0	4
	2–3 months	2	2.2% Fe citrate + 0.1% $CuSO_4 \cdot 5H_2O$	−27	1,344	105–1,870	32	6.8	3.5
		3	0.3% Choline	55	246	41–1,220	29	1.0	0
		4	0.3% Choline + 0.1% $CuSO_4 \cdot 5H_2O$ + 2.2% Fe citrate	−28	2,400	2,300–2,500	46	1.3	0
III	7/group	1	None	61	12	9–21	25	45.2	3
	8 months	2	0.1% $CuSO_4 \cdot 5H_2O$	−50	156	17–300	67	33.3	2.2
		3	0.3% Choline	107	25	11–40	74	15.5	1
		4	0.1% $CuSO_4 \cdot 5H_2O$ + 0.3% choline	−111	298	28–1,000	116	19.4	1
IV	4/group	1	0.1% $CuSO_4 \cdot 5H_2O$ + 5% choline + 10% casein	22	147	117–198	68		0.3
	2½ months	2	0.3% $CuSO_4 \cdot 5H_2O$ + 5% choline + 10% casein	−31	158	143–181	65		0
		3	0.5% $CuSO_4 \cdot 5H_2O$ + 5% choline + 10% casein	−61	230	169–342	100		0

[1] Titratable fatty acids calculated as palmitic expressed as per cent of fresh liver in experiments I and II; total ether extract as per cent of dried livers in experiment III.

[2] Arbitrary scale in which zero is normal and 4 is maximum visible fat.

choline fed. The weight loss with 0.1% copper and 1.0% choline was the same as that obtained with 0.5% copper without a choline supplement. It will be noted, however, that in this experiment (table 4) an interaction of choline and copper with respect to weight loss was not significant. Since choline can scarcely be considered a toxic addition to a choline-deficient diet, and a statistically significant interaction with respect to weight loss was apparent in experiment III, this result must be interpreted as showing that the interaction of

TABLE 2

Summary table showing the results of variance analyses of the weight gains in experiments I, II and III

EXPERIMENT	EFFECT STUDIED	SIGNIFICANCE
I	Iron supplementation	Not significant
	Copper supplementation	Not significant
	Interaction (iron with copper)	Significant at 5% level
II	Choline supplementation	Not significant
	Fe and Cu supplementation	Significant at 5% level
	Interaction (Fe and Cu with choline)	Significant at 5% level
III	Copper supplementation	Highly significant [1]
	Choline supplementation	Highly significant [1]
	Interaction (Cu with choline)	Highly significant [1]

[1] Less than one chance in 100 that the results are due to chance.

copper and choline occurs at lower levels of copper (i.e., between 0 and 0.1%) than were used in this experiment. It would be of interest to determine at what copper concentration choline becomes a detrimental agent.

The results of the chemical analyses from experiment III follow the same trends shown by the first two experiments; namely, an increase in liver copper when choline was fed and an increase in liver iron and decrease in liver fat when copper was fed. None of the changes was statistically significant, however, other than those obviously expected. (Liver copper rises with copper feeding and the fat decreases when choline is fed.) In the larger experiment V, the results were more

instructive (tables 3 and 4). Here the feeding of choline definitely raised liver copper and the statistical interaction of copper and choline was significant at the 5% level of probability. Both choline and copper feeding definitely raised the level of liver iron. Fat analyses were not made, although

TABLE 3

The effect of various levels of copper and choline on weight gain and liver composition in experiment V

LEVEL OF CHOLINE	MEASURE	LEVEL OF $CuSO_4 \cdot 5H_2O$ IN DIET			AVERAGE, ALL LEVELS OF Cu
		0.1%	0.3%	0.5%	
None	Weight gain, gm	—30	—58	—98	—62
	Liver Cu, μg/gm dry	101	201	196	183
	Liver iron, mg/100 gm dry	45	85	68	65
	Liver fat, histological	2.9	1.9	1.8	2.2
	Liver iron, histological	1.3	2.0	1.5	1.7
0.5% choline chloride	Weight gain, gm	—44	—76	—103	—74
	Liver Cu, μg/gm dry	215	279	933	476
	Liver iron, mg/100 gm dry	82	104	120	102
	Liver fat, histological	0.3	0.3	0.1	0.23
	Liver iron, histological	1.0	1.5	1.9	1.5
1.0% choline chloride	Weight gain, gm	—93	—141	—144	—126
	Liver Cu, μg/gm dry	283	270	523	359
	Liver iron, mg/100 gm dry	114	124	116	118
	Liver fat, histological	0	0	0	0
	Liver iron, histological	2.1	2.1	1.8	2.0
Average, all levels of choline	Weight gain, gm	—56	—92	—115	
	Liver Cu, μg/gm dry	200	250	552	
	Liver iron, mg/100 gm dry	81	104	102	
	Liver fat, histological	1.1	0.7	0.6	
	Liver iron, histological	1.5	1.8	1.8	

the histological results showed changes in the same direction as those observed in the earlier experiments.

The results of experiment IV (table 1) were obtained with animals from the same lot, and at the same time, as experiment V. The results may therefore be compared with those in table 3. It is apparent that even with the very high level of choline used, 5%, casein had a marked protective effect.

The weight loss was much less than that obtained with 1% choline at each level of copper. Liver copper and liver iron were also greatly reduced by the addition of casein to the diet.

All animals which received the basal diet, low in choline and protein, showed those histologic changes described in choline deficiency. Fatty vacuolization of liver cells was extensive after 8 or more weeks and an occasional cell was necrotic. The livers were congested, and in severely deficient animals occasional focal hemorrhages and collections of lymphocytes were noted. There was no scarring except after long periods of deficiency (experiment III). Hemosiderin

TABLE 4

Results of variance analysis of the data from experiment V

EFFECT STUDIED	GAIN IN WEIGHT	LIVER COPPER	LIVER IRON	SIGNIFICANCE LEVEL [2]	
				5%	1%
	F [1]	F [1]	F [1]		
Copper supplementation	4.53	9.70	7.46	3.35	5.49
Choline supplementation	5.07	6.60	29.3	3.35	5.49
Interaction (copper with choline)	0.15	3.31	1.40	2.73	4.11

[1] Variance ratio; see G. W. Snedecor, "Statistical Methods," 4th ed., Iowa State College Press, Ames, 1946.

[2] F values larger than these indicate significance at the indicated probability levels.

was present in occasional Kupffer cells and in rare liver cells. The results of examining sections of the lungs, heart, spleen, adrenals and kidneys were essentially negative.

The livers of animals receiving the basal diet to which copper had been added showed essentially the same changes, although in experiments I and III the fatty changes were not as severe as in the control animals (table 1).

Iron supplements appeared to have a detrimental effect. Scarring was seen in most of the animals fed this supplement and in some cases it was extensive. Fatty changes may have been slightly more severe than in the control animals. Occasional Kupffer and parenchymal cells contained stainable

iron. Large quantities of iron were demonstrated in the spleen and occasionally in the cells lining the kidney tubules. Macrophages containing lipoid material similar to those in the liver were found in the lungs.

Animals receiving both iron and copper generally had less fat infiltration than control animals. Scarring was seen in three livers but was not extensive. The degree of iron deposition was similar to that seen in the animals which received iron alone.

The addition of choline or casein, or both, to the diet largely prevented fat infiltration in the liver. All tissues appeared normal.

DISCUSSION

It will be appreciated that the time allowed for the experiments, the basal diet, the levels of the supplements, and so forth, all had to be arbitrarily chosen when these studies were started. Other conditions or levels of supplementation might produce more striking effects. It was also apparent after the first experiments that the number of variables was large and that the possible interrelations could not be delineated in a few experiments of convenient size. It was necessary to select the variables for separate study. This paper is essentially a study of the copper-choline relationship, which appears to be the most clear-cut at the present time.

In all of the studies the combination of copper and choline caused a much greater loss in weight than the same level of copper alone when compared to the weight of appropriate control animals. Choline alone was, of course, clearly beneficial, as would be expected with a choline-deficient, low protein diet. Choline supplementation also increased the level of liver copper. In the more carefully planned experiments in which larger numbers of animals were used, all of these relationships were highly significant. Significant changes in liver fat and liver iron as the result of these two supplements were also observed.

While the data are not sufficient to allow clear interpretation of all the results obtained, the following mechanisms appear the most obvious and most logical at the present time. Choline apparently increases the absorption or deposition, or both, of copper in the liver, or inhibits its excretion. Any one of these would account for the detrimental action of choline in the presence of copper, although the mechanism is entirely unknown. Until other data are available, the changes in liver iron and fat may be considered as purely passive and the result of the toxic action of copper and choline. It is known that starvation or weight loss increases liver iron (Kinney et al., '49) and starvation will also decrease the level of fat in the livers of choline-deficient animals (Best and Ridout, '38). These suggestions do not preclude the apparent interrelationship of copper and iron observed in experiment I when a high-iron diet was also fed.

Casein feeding minimizes the effects of copper, although the quantitative aspects of this action with regard to copper and choline have not been investigated. In view of the fact that liver copper was lower after feeding casein, it seems probable that casein affects either the absorption or excretion of copper.

The first experiment indicated an interaction between iron and copper, since the combined supplement was much more detrimental than either alone. In work published since these studies were started (Kinney et al., '49) it was shown that the level of iron used had little effect on growth when the diet contained the 4% salt mixture used in the present diet. Also, iron toxicity was associated with much higher levels of liver iron than were found in these animals. Since this interaction did not reach a high level of significance, the present data must be considered as suggestive until confirmation is available.

As is indicated in our introduction to this paper, the present experiments did not produce in the animals a condition closely resembling hemochromatosis. The original hypotheses are not supported by the data. The increase in liver iron which

occurs during copper poisoning can be most easily explained as the result of the loss in weight suffered by the animals.

CONCLUSION

It has been shown that the addition of choline to a choline-deficient, low protein diet containing moderately toxic levels of copper *increases* the detrimental effect of the diet as measured by weight loss. This effect is quantitative in that high levels of choline caused greater losses of weight than low levels. Choline also increases the level of liver copper under these conditions. It is concluded that choline either increases the absorption or the deposition, or both, of copper, or inhibits its excretion.

Copper and choline feeding also increase the level of liver iron and decrease the level of liver fat. Until further evidence is available, these changes can probably be considered secondary to the loss in weight caused by the copper and choline feeding.

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RELATIONSHIPS BETWEEN INTAKE AND SERUM LEVELS OF ASCORBIC ACID, VITAMIN A, AND CAROTENE OF SELECTED GROUPS OF CHILDREN WITH PHYSICAL SIGNS OF VITAMIN DEFICIENCIES[1]

SUSAN B. MERROW,[2] R. F. KRAUSE,[3] J. H. BROWE,[4] C. A. NEWHALL AND H. B. PIERCE[5]

Departments of Biochemistry and Anatomy, College of Medicine, University of Vermont, Burlington

TWO FIGURES

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INTRODUCTION

Nutrient intake and blood serum levels are frequently determined in nutrition studies of population groups (Aykroyd et al., '49; Bessey and Lowry, '47; Nutrition Reviews, '50; Pierce et al., '45; Putnam et al., '49; Storvick et al., '51a, b). The practical value of such information lies in its interpretation and use in the diagnosis, treatment, and prevention of malnutrition. There is still need for information concerning the interpretation of nutrient intake and blood determinations. The purpose of this study was to determine

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[2] Present address: Department of Home Economics, University of Vermont, Burlington.

[3] Present address: Department of Biochemistry, School of Medicine, University of West Virginia, Morgantown.

[4] Present address: Bureau of Nutrition, State of New York Department of Health, Albany.

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whether data, collected by methods to be described and under conditions which are believed to be fairly typical of population studies, would reveal any meaningful relationships between nutrient intake and serum levels and thereby contribute to a better understanding of these two types of nutrition data.

EXPERIMENTAL

The 160 subjects concerned were part of a total group of 329 male and female children, ages 8 to 15, attending public and parochial schools in Burlington, Vermont. All showed the widely accepted anatomical changes considered to be related to deficiencies of vitamin A, riboflavin, niacinamide and/or ascorbic acid (Council on Foods and Nutrition, '46; Jolliffe et al., '50; Browe and Pierce, '50). The children studied had been grouped on the basis of progress and severity of lesions, and sex and age, and then divided into therapy and non-therapy sections in order to conduct a study of the effect of therapy with a single vitamin upon blood chemistry and lesions. The data reported in this paper pertain to members of the non-therapy group.

In examining tissues for vitamin A deficiency, the bulbar conjunctivae were examined for loss of transparency, increase in vascularity and thickening; the palpebral conjunctivae for hypertrophied follicles; the skin for dryness, scaling and crinkling (xerosis), and plugging of the hair follicles on the postero-lateral aspects of arms and the lateral aspects of thighs. The tongue, believed to be affected in niacin deficiency, was examined for redness, swelling, changes in papillae, and fissuring. Redness, swelling, hypertrophy, fusion, and atrophy of the papillae were particularly noted. Recorded as abnormalities suggestive of ascorbic acid deficiency were thickening, hardness, redness, swelling, tenderness and bleeding of the gums on pressure, recession of marginal gingivae and interdental papillae, and retraction of the gums from the teeth. As regards changes believed to be descriptive of riboflavin deficiency, the lips were examined for increased redness, wrin-

kling with lack of normal pattern, or swelling and loss of wrinkling. Also redness, fissuring, maceration and scarring of the angles of the mouth were noted, as well as redness and greasiness of the nasolabial folds, outer canthi and areas behind the ears.

It is the intention of the authors to limit this presentation to a study of the relationships beween nutrient intake and serum levels of these children, since an analysis of the physical signs will be reported in a subsequent paper.

When data for the entire control group were examined, relationships between nutrient intake and serum levels were inconclusive. Therefore, subjects were selected who: (a) had a general pattern of low or of high serum levels; or (b) a general pattern of low or of high nutrient intakes. Due to circumstances beyond our control, it was impossible to secure 100% of the blood and nutrient determinations on all subjects over the three-year period. Ninety-eight per cent had 7 or 8 blood determinations and 80% provided 6 to 9 24-hour food intake records.

In this paper we present, first, relationships between serum levels and nutrient intakes for groups of children from the control subjects having the "highest" or "lowest" serum levels. Second we show relationships between nutrient intakes and serum levels for selected groups of children having the "highest" or "lowest" nutrient intakes.

In selecting the cases for the "high" and "low" serum groups, the scale of blood levels according to Bessey and Lowry ('47) was used. Thus, if the general pattern of the serum determinations of an individual subject was in the range of good or excellent, he was placed in the "high" group, and if in the poor or fair range, he was placed in the "low" group. We had three groups of pairs selected from the control subjects having the "highest" or "lowest" serum levels; first, of ascorbic acid[6]; second, of vitamin A; and third,

[6] All children in the ascorbic acid serum level groups exhibited widely accepted physical signs of ascorbic acid deficiency.

of carotene.[7] Table 1 presents the distributions, ranges, and medians of the serum levels of these groups and demonstrates the distinct differences as well as the degree of overlapping that occurred.

In selecting the cases for the "high" and "low" nutrient intake groups, those were chosen whose diets showed a general pattern of high or low values. Although the general pattern was high or low, inspection of the ranges reveals the wide variability characteristic of this type of nutrition data. Four groups of pairs were selected as having the "highest" or "lowest" nutrient intake; first, of ascorbic acid[8]; second, of vitamin A plus carotene; third, of vitamin A[9] alone; and fourth, of carotene[10] alone. Table 2 presents the distributions, ranges, and medians of the nutrient intake groups and demonstrates the distinct differences as well as the degree of overlapping that occurred. The assumption was made that animal food contains only vitamin A and plant food only the vitamin A-active carotenoids.

It is essential to remember that the terms "high" or "low" are purely relative; that is, they refer only to the higher or lower values in the range of the data collected for these particular children.

Serum levels, determined by the micro-chemical techniques of Bessey et al. (Lowry et al., '45; Bessey et al., '46), were obtained in the spring and fall of 1946 and the winter, spring, and fall of 1947 and 1948. Nutrient intakes, calculated by use of the standard tables (Bureau of Human Nutrition and Home

[7] All children in the vitamin A and carotene serum level groups exhibited the widely accepted physical signs of vitamin A deficiency except one subject in the "high" vitamin A serum level group. This subject had physical signs of ascorbic acid, niacin, and riboflavin deficiencies.

[8] All children in the ascorbic acid nutrient intake groups exhibited the widely accepted physical signs of ascorbic acid deficiency.

[9] All children in the vitamin A, carotene, and vitamin A plus carotene nutrient intake groups exhibited the widely accepted physical signs of vitamin A deficiency except one subject in the "low" vitamin A intake group who had signs of ascorbic acid, niacin, and riboflavin deficiencies.

[10] See footnote 9. This same subject was in the "high" carotene nutrient intake group.

TABLE 1

Distribution of serum levels for serum level groups

Range	ASCORBIC ACID		Range	VITAMIN A		CAROTENE	
	"High"	"Low"		"High"	"Low"	"High"	"Low"
mg%	no.	no.	*μg%*	no.	no.	no.	no.
0.0–0.1		36	0– 4				
0.2–0.3	3	61	5– 9		6		
0.4–0.5	4	22	10–14		13		
0.6–0.7	8	18	15–19	1	29		
0.8–0.9	11	3	20–24		27		3
1.0–1.1	13	3	25–29	4	14		3
1.2–1.3	22		30–34	11	7		3
1.4–1.5	13		35–39	14	2		8
1.6–1.7	15		40–44	20	1		13
1.8–1.9	8		45–49	15			14
2.0–3.1	12		50–54	6			15
			55–59	6			14
			60–64	1			19
			65–69				11
			70–74				10
			75–79			1	9
			80–84				4
			85–89			1	7
			90–94				6
			95–99			1	3
			.....				
			100–124			20	1
			125–149			33	
			150–174			24	
			175–199			12	
			200–224			9	
			225–249			7	
			250–274			6	
			275–299			3	
			300–354			1	
Total number							
Determinations	109	143		78	99	118	143
Subjects	14	19		10	13	15	19
Range [1]	0.2–3.1	0.02–1.1		18–64	6–40	75–354	22–112
Median [1]	1.34	0.32		43	20	155	62

[1] Ascorbic acid in mg%. Vitamin A and carotene in μg%.

TABLE 2

Distribution of nutrient intakes for nutrient intake groups

Range	ASCORBIC ACID		Range	VIT. A + CAROT.		VITAMIN A		CAROTENE	
	"High"	"Low"		"High"	"Low"	"High"	"Low"	"High"	"Low"
mg	no.	no.	*100 I.U.*	no.	no.	no.	no.	no.	no.
0– 9		20	0– 4		2		17	3	133
10– 19		55	5– 9		12		54	17	21
20– 29		48	10–14		14		85	30	6
30– 39		30	15–19		19	2	80	17	2
40– 49	21	4	20–24		23	7	46	14	1
50– 59	17		25–29	1	17	11	4	8	
60– 69	21		30–34	12	4	21		6	1
70– 79	12		35–39	24	2	22			
80– 89	11		40–44	19	3	28		3	
90– 99	10		45–49	22	2	12		4	2
100–109	11		50–54	14		8		4	1
110–119	10		55–59	15		7		5	
120–129	13		60–64	11	3	8		7	
130–139	4		65–69	9		3		2	
140–149	2		70–74	10				2	
150–159	2		75–79	7	1	1		1	
160–169	1		80–84	2	1			2	
170–179	5		85–89	7		1		4	
180–189	2		90–94	6					2
190–199	2		95–99	3	1			4	
200–421	4		.....						
			100–124	14	2	3		7	1
			125–149	13		1		7	
			150–174	9				3	
			175–199	5		2		2	
			200–375	18	1	4		4	
Total number									
Determinations	148	157		221	107	141	286	156	170
Subjects	19	22		26	14	18	37	22	23
Range [1]	41–421	3–41		27–375	1–251	18–369	0–25	0–311	0–115
Median [1]	84	21		62	22	42	14	25	2

[1] Ascorbic acid in mg. Vitamin A and carotene in 100 I.U.

Economics, '45), were determined in the winter, spring, and fall of the years 1946, 1947, and 1948.

A report of the foods eaten during the preceding 24 hours was secured by an interview with each subject. A special technique was developed in order to obtain as valid a record as possible. The interview was conducted under circumstances which would minimize motives that might tempt the subject to modify the report of the actual intake. The purpose of our study was carefully explained and ample opportunity was allowed for questions and discussion. An estimate of the amounts of foods ingested was facilitated by the use of food models, dishes, and utensils similar to those used by the subjects. Suggestion was avoided by using the models only after the record was obtained.

RESULTS

Table 3 shows the medians, ranges, and number of nutrient intakes computed for the "high" and "low" serum level groups.

Table 4 shows the medians, ranges, and number of serum determinations for the "high" or "low" nutrient intake groups.

Figure 1 shows the nutrient intakes of the "high" and "low" serum level groups. The "high" groups, designated 1, represent the subjects with the high, and the "low" groups, designated 2, the subjects with the low serum level. For ascorbic acid, group 1 had a median serum level of 1.34 mg % (range 0.2–3.1 mg %). Seventy per cent had an ascorbic acid intake of 40 mg or over. In group 2, with a median serum level of 0.32 mg % (range 0.02–1.1 mg %), only 33% had an ascorbic acid intake of 40 mg or over. An analysis made on the significance of difference in percentage distribution (Hill, '48) between the "high" and "low" groups with respect to intakes of 40 mg and over shows this difference, 70% versus 33%, to be significant.

The second set of bars in figure 1 presents nutrient intake of vitamin A plus carotene versus serum levels of vitamin A.

TABLE 3

Nutrient intakes of serum level groups

SERUM LEVEL Subjects	Group	NUTRIENT INTAKES Median	Median	Range	Diets
	Ascorbic acid	Group ≧ 40 mg	*Ascorbic acid* Group < 40 mg		
no.		*mg*	*mg*	*mg*	*no.*
14	1. "High"	79 (86)[1]	29 (38)[1]	1–216	124
19	2. "Low"	65 (55)	23 (112)	2–233	167
	Vitamin A	Group ≧ 3,000 I.U.	*Vitamin A plus carotene* Group < 3,000 I.U.		
no.		*I.U.*	*I.U.*	*I.U.*	*no.*
10	1. "High"	5,500 (66)	2,390 (22)	300–23,100	88
13	2. "Low"	4,950 (61)	1,890 (52)	200–37,700	113
	Vitamin A	Group ≧ 3,000 I.U.	*vitamin A* Group < 3,000 I.U.		
no.		*I.U.*	*I.U.*	*I.U.*	*no.*
10	1. "High"	3,780 (28)	2,190 (60)	700–18,100	88
13	2. "Low"	3,970 (33)	1,730 (80)	200–20,800	113
	Carotene	Group ≧ 2,000 I.U.	*Carotene* Group < 2,000 I.U.		
no.		*I.U.*	*I.U.*	*I.U.*	*no.*
15	1. "High"	8,400 (54)	860 (79)	0–20,100	133
19	2. "Low"	4,130 (41)	380 (118)	0–36,100	159

[1] Numbers in parentheses indicate number of diet determinations.

TABLE 4

Serum levels of nutrient intake groups

NUTRIENT INTAKE		SERUM LEVELS			
Subjects	Group	Median	Median	Range	Serums
	Ascorbic acid	Group ≧ 0.6 mg%	*Ascorbic acid* Group < 0.6 mg%		
no.		mg%	mg%	mg%	no.
19	1. "High"	1.31 (91)[1]	0.48 (38)[1]	0.08–2.64	129
22	2. "Low"	1.14 (65)	0.29 (68)	0.05–2.03	133
	Vit. A + carotene	Group ≧ 30 μg%	*Vitamin A* Group < 30 μg%		
no.		μg%	μg%	μg%	no.
26	1. "High"	39 (127)	23 (65)	7–63	192
14	2. "Low"	40 (48)	24 (45)	12–72	93
	Vitamin A	Group ≧ 40 μg%	*Vitamin A* Group < 40 μg%		
no.		μg%	μg%	μg%	no.
18	1. "High"	46 (28)	30 (91)	10–56	119
37	2. "Low"	47 (54)	27 (188)	8–72	242
	Carotene	Group ≧ 80 μg%	*Carotene* Group < 80 μg%		
no.		μg%	μg%	μg%	no.
22	1. "High"	153 (114)	57 (16)	34–354	130
23	2. "Low"	110 (66)	56 (80)	22–182	146

[1] Numbers in parentheses indicate number of serum determinations.

In group 1, with a median serum level of 43 μg % (range 18–64 μg %), 76% had a vitamin A plus carotene intake of 3,000 I.U. and over. In group 2, with median serum level of 20 μg % (range 6–40 μg %), 54% had a nutrient intake of 3,000 I.U. and over. The difference between the percentage dis-

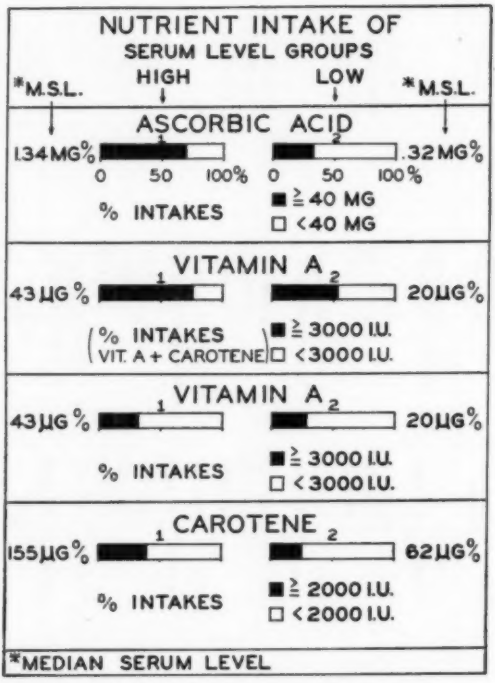

Fig. 1 Nutrient intake of serum level groups.

tribution of the two groups is significant. Hence in the group with the "high" serum levels there were significantly more nutrient intakes of 3,000 I.U. and over than in the "low" serum level groups.

From the third set of bars in figure 1 we see that 32% of group 1, median serum level of 43 μg % (range 18–64 μg %), had a nutrient intake of 3,000 I.U. and over, whereas 29% of

group 2, median serum level of 20 μg % (range 6–40 μg %), had a vitamin A intake of 3,000 I.U. and over. In contrast with the positive relationship shown for nutrient intake of ascorbic acid and vitamin A plus carotene, there is in this case no significant difference in the percentage distribution of nutrient intakes of 3,000 I.U. and over between the "high" and "low" serum level groups. In other words, intake of vitamin A per se is not reflected by the vitamin A serum levels.

When we compare the nutrient intake and serum levels of carotene, as shown in the last set of bars in figure 1, we find that in group 1, median serum level of 155 μg % (range 75–354 μg %), 40% of the nutrient intake records were 2,000 I.U. and over, whereas in group 2, median serum level of 62 μg % (range 22–112 μg %), only 25% had a nutrient intake of 2,000 I.U. and over. This difference in percentage distribution is significant.

Figure 2 presents the serum levels of the "high" and "low" nutrient intake groups. The "high" groups, designated 1, had the high nutrient intakes and the "low" groups, designated 2, the low nutrient intakes. The top bars show the percentage distribution of serum levels of ascorbic acid over and under 0.6 mg %. Group 1 had a median intake of 84 mg (range 41–421 mg). Seventy-one per cent had serum levels of 0.6 mg % and over. In group 2, with a median intake of 21 mg (range 3–41 mg), 49% had serum levels of 0.6 mg % and over. The difference between the percentage distribution in these two groups is significant; that is, in the group with the "high" nutrient intake there were more serum levels of 0.6 mg % and over than in the "low" nutrient intake group.

The second set of bars presents data for vitamin A. In group 1, with a median nutrient intake of 6,180 I.U. (range 2,700–37,500 I.U.), 67% had a vitamin A serum level of 30 μg % and over, while group 2, median nutrient intake of 2,160 I.U. (range 100–25,100 I.U.), had 51% with a vitamin A serum level of 30 μg % and over. This difference in percentage distribution also proves significant, and indicates

that there were more serum levels of 30 μg % and over in the "high" than in the "low" nutrient intake group.

From the third set of bars we find that for vitamin A intake per se there is no significant difference in the percentage distribution of serum levels of 40 μg % and over. For the

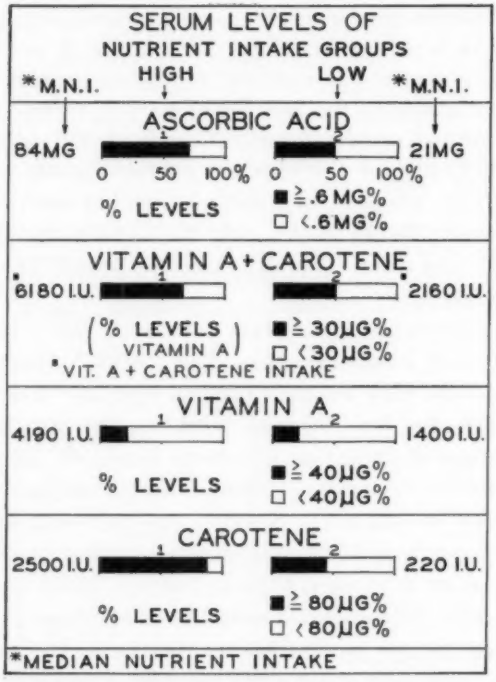

Fig. 2 Serum levels of nutrient intake groups.

values obtained, serum levels do not reflect vitamin A intake per se. This result is similar to that obtained for vitamin A intake per se of the "low" and "high" serum level groups, as shown previously in figure 1.

The 4th set of bars, depicting groupings on the basis of nutrient intake for carotene, shows that in group 1, with a median nutrient intake of 2,500 I.U. (range 0–31,100 I.U.),

88% had serum levels of 80 μg % and over, while in group 2, with a median nutrient intake of 220 I.U. (range 0–11,500 I.U.), only 45% had serum levels of 80 μg % and over. This difference in percentage distribution of serum levels is significant.

SUMMARY AND CONCLUSIONS

A three-year study has been made of serum levels and nutrient intake of a group of male and female school children.

Under the conditions of this investigation and for the values shown, the nutrient intakes of ascorbic acid, total vitamin A, and carotene reflect serum levels of ascorbic acid, vitamin A, and carotene, respectively. Serum levels of ascorbic acid, vitamin A, and carotene reflect nutrient intake of ascorbic acid, total vitamin A, and carotene, respectively. Relationships were not found between serum levels of vitamin A and nutrient intake of vitamin A per se.

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TOCOPHEROL CONTENT OF HUMAN MILK AND OF COWS' MILK PRODUCTS USED FOR INFANT FEEDING [1]

PHILIP L. HARRIS, MARY L. QUAIFE AND PATRICIA O'GRADY

Research Laboratories, Distillation Products Industries, Division of Eastman Kodak Company, Rochester, N. Y.

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It is well established that mammalian newborn, whether full-term or premature, are poorly endowed at birth with respect to the vitamin E content of body tissues (Varangot et al., '44; Whiting and Loosli, '48; Mason and Bryan, '40). Wright and co-workers ('51) have recently studied the effect of the type of feeding regime on the vitamin E status of human newborn. They found that the plasma tocopherol level, 0.4 mg% at birth, increased to 1.0 to 1.5 mg% within 6 days in breast-fed infants, presumably due to the high tocopherol content of ingested colostrum. In formula-fed infants the plasma level rose slowly and was only 0.75 mg% at 8 months of age. Premature infants, kept on a hospital formula, showed a steady decrease in the level of plasma tocopherols to less than 0.1 mg% at 40 days.

Existing data relative to the tocopherol content of human milk, and of milk products ordinarily used for infant feeding, are somewhat meager. Consequently, a variety of these have been chemically analyzed for vitamin E content. They include human milk samples, representing different stages of lactation, and samples of powdered and of evaporated cows' milk as well as of proprietary infant foods. The results obtained are compared with existing data on the vitamin E content of infant foods.

[1] Communication 184.

METHODS

Total tocopherols in fluid milks were measured by the method of Quaife ('47). This value represents chiefly α-tocopherol, since mammals selectively absorb and deposit this form of tocopherol in their tissues (Lundberg et al., '47; Quaife et al., '49). The fat content of fluid milks was measured by the Babcock method as adapted for small (5-ml) samples (Kolmer and Boerner, '45).

Proprietary infant foods were analyzed for the total and for γ- + δ-tocopherols, as well as fat content, by the method of Quaife and Harris ('48). The difference between total and γ- + δ-tocopherol content is taken to represent α-tocopherol content. Only total tocopherols were measured in powdered cows' milk by this method (Quaife and Harris, '48); this value probably represents α-tocopherol.

RESULTS AND DISCUSSION

Human milk

A series of human milk samples from various donors, representative of the third to 37th weeks of lactation, were analyzed for vitamin E and fat content. A portion of each sample had been pasteurized. These samples had been shipped and received, frozen, within a week of collection.[2] They were stored at — 20°C. and analyzed within a month's time. The tocopherol and fat contents of these 20 milk samples are given in table 1 in order of stage of lactation.

There is no apparent dependence of tocopherol content on stage of lactation within the period three to 37 weeks. Values range, at random, from 0.10 to 0.48 mg tocopherols/100 ml of milk.

The mean value for the 10 raw milk samples is 0.238 ± 0.034 and for the pasteurized samples 0.242 ± 0.031 mg tocopherols/100 ml of milk. Thus, pasteurizing caused no loss of tocopherol in these milk samples.

[2] Obtained from the Mothers' Milk Bank, Inc., San Francisco, California, through the courtesy of Mrs. A. E. Graupner, President.

The correlation coefficient for the tocopherol and the fat content was calculated for the human raw milk samples. A value for "r" of + 0.82 with a standard error of ± 0.334 was obtained. This value is significant (p = 0.05 to 0.02) according to the "t" test, indicating that the tocopherol concentration is correlated with the fat content of the milk.

The mean tocopherol content of human milk fat in the present series (80 μg/gm) is considerably higher than was found for human milk previously in this laboratory (37 to 58 μg/gm; Quaife, '47). The present samples were analyzed when fresh — the others after a year's storage.

TABLE 1

Total tocopherol and fat content of human milk samples

WEEK OF LACTATION	TOCOPHEROL CONTENT (MG/100 ML)		FAT CONTENT (%)	
	Raw	Pasteurized	Raw	Pasteurized
3	0.23	0.26	2.9	2.9
7	0.28	0.19	3.2	3.2
8	0.17	0.18	2.4	2.9
11	0.48	0.46	4.5	4.2
11	0.17	0.23	3.4	3.4
14	0.26	0.24	3.2	2.0
18	0.21	0.21	3.4	2.2
21	0.10	0.14	2.5	1.8
30	0.14	0.17	2.1	2.4
36	0.34	0.36	5.4	4.8
Mean ± S.E.	0.238 ± 0.034	0.242 ± 0.031	3.3 ± 0.3	3.0 ± 0.3

A composite sample from a milk bank, stored 9 months or more, was also obtained for this study.[3] It contained 0.141 mg tocopherols/100 ml of milk, or 49 μg/gm of milk fat. This lower value may also be due to loss during storage.

All of these tocopherol values for human milk (mean of a series, or assay values of composite samples) are higher (up to 4 times) than the tocopherol content of ordinary cows' milk. Values for the latter were found by Swanson

[3] From the Dispensary for Mothers' Milk, Louisville, Kentucky, through the courtesy of Mrs. J. L. Herin, Secretary.

TABLE 2

Tocopherols in cows' milk products and proprietary infant foods

PRODUCT	BRAND	SAMPLE NO.	TOTAL TOCOPHEROLS		α-TOCOPHEROL [1]		FAT CONSTITUENTS, AS LABELLED
			mg%	*mg/gm fat*	*mg%*	*mg/gm fat*	
Evaporated cows' milk	A	1	0.25	0.031	...	....	....
Evaporated cows' milk	A	2	0.25	0.031	...	....	....
Evaporated cows' milk	B	1	0.20	0.025	...	....	....
Evaporated cows' milk	B	2	0.18	0.023	...	....	....
Powdered whole cows' milk	C	1	0.92	0.034	...	....	....
Powdered whole cows' milk	C	2	1.01	0.038	...	....	....
Powdered whole cows' milk	C	3	1.06	0.040	...	....	....
Fluid infant food	D	1	1.82	0.243	...	....	Beef fats; coconut, corn, and soybean oils; soybean lecithin.
Powdered infant food	E	1	4.19	0.165	2.83	0.111	Coconut, palm, and peanut oils; lecithin.
Powdered infant food	E	2	5.66	0.222	4.34	0.171	Coconut, palm, and peanut oils; lecithin.
Powdered infant food	F	1	5.86	0.226	2.60	0.115	Beef fats; coconut, corn, and soybean oils; soybean lecithin.
Powdered infant food	F	2	4.63	0.188	1.13	0.046	Beef fats; coconut, corn, and soybean oils; soybean lecithin.
Powdered infant food	F	3	5.21	0.188	2.25	0.081	Beef fats; coconut, corn, and soybean oils; soybean lecithin.
Powdered infant food	G	1	2.72	0.110	0.73	0.029	Coconut, corn, and olive oils.

[1] The value for α-tocopherol in ordinary cows' milk is presumably the same as that for total tocopherols.

TABLE 3

TABLE 3

Reported values for the tocopherol content of milk and milk products

PRODUCT	NO. OF SAMPLES	TOCOPHEROL CONTENT		REFERENCE
		mg/100 ml or 100 gm	*μg/gm fat*	
Human milk:				
Date of collection unspecified	3	0.5–3.6	(167–1,200)[1]	Kofler, '43
Date of collection unspecified	..	0.25–3.0	(83–1,000)[1]	Neuweiler, '48
Colostrum	15	0.13–3.6	76–1,800	Quaife, '47
Colostrum (2nd–3rd day)	2	2.6–3.3	(867–1,100)[1]	Abderhalden, '47
Colostrum (4th–10th day)	12	0.40–1.85	(133–617)[1]	Abderhalden, '47
range			76–1,800	
Mature milk (1st–8th month)	4 (composites of 14)	0.11–0.15	37–58	Quaife, '47
Mature milk (3rd–37th week)	10	0.10–0.48	25–107	Present paper
range			25–107	
Cows' milk:				
Fluid, unpasteurized (winter)	..	0.08–0.15	17–30	Swanson, quoted in Quaife, '47
Fluid, unpasteurized (summer)	..	0.17	42	Harris et al., '47
Fluid	..	0.056	(14)[1]	Lieck and Willstaedt, '45
Fluid	..	0.06	(15)[1]	Abderhalden, '47
Fluid	..	0.05–0.15	(13–38)[1]	Neuweiler, '48
Fluid	..	..	10–45	Bird et al., '51
Fluid	..	0.020	5	Emmerie and Engel, '43
Fluid (representing 13 states)	15	..	25	Handwerk and Bird, '51
Fluid (Guernsey, Holstein, Brown Swiss, and Jersey)	128	..	22–30	Krukovsky et al., '50
Fluid	..	..	8–27	Parrish et al., '47
range			5–45	
Evaporated	4	0.18–0.25	23–31	Present paper
Powdered whole	3	0.92–1.06	34–40	
Goats' milk:				
Fluid	13	..	14–31	Whiting and Loosli, '48
Proprietary infant foods:[2]				
Powdered	6	0.73–4.34	29–171	Present paper

[1] Estimated on the basis of 3% fat content for human milk and 4% fat content for cows' milk.

[2] Values are for α-tocopherol content.

to range from about 15 to 40 μg/gm, depending in part on the season of the year (Quaife, '47).

Evidently, human milk contains two to 4 times as much tocopherol as does cows' milk.

Milk products and proprietary infant foods

Infants in the United States who are not breast-fed commonly receive modified cows' milk or proprietary products which are designed to resemble human milk in essential nutrients. The vitamin E content of some typical products has been determined, and values are listed in table 2.

The tocopherol content of these products can be compared, based on fat content. The evaporated milks have a tocopherol content in fat ranging from 23 to 31 μg/gm. The proprietary milk substitutes have an α-tocopherol content, in terms of fat content, varying from 29 to 171 μg/gm.

In table 3 are listed all available pertinent data on the tocopherol content of milk or milk products commonly used in feeding newborn infants. Apparently human colostrum, human milk, and cows' or goats' milk, in descending order, provide varied amounts of vitamin E both on a fluid milk or fat content basis. Proprietary baby foods, compared as to α-tocopherol content per unit weight of fat, provide highly variable amounts of vitamin E, but usually much less than does human colostrum.

SUMMARY

1. The tocopherol content of a series of human milk samples representing various donors and stages of lactation was 0.10 to 0.48 mg/100 ml of milk, with a mean of 0.24 mg/100 ml, or about 80 μg/gm of fat.

2. Tocopherol values did not vary with stage of lactation (third through 37th week) but were correlated with the fat content of the milk.

3. No loss of tocopherol occurred on pasteurizing human milk.

4. Evaporated cows' milk and samples of whole milk powder contained 23 to 40 μg of tocopherol/gm of fat.

5. Proprietary infant foods contained 29 to 171 μg of α-tocopherol/gm of fat.

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GROWTH RESPONSE AND VITAMIN B_{12} TISSUE LEVELS IN VITAMIN B_{12}-DEFICIENT RATS AND CHICKS FED RIBOFLAVIN, 5,6-DIMETHYLBENZIMIDAZOLE AND RELATED COMPOUNDS [1]

J. M. COOPERMAN, B. TABENKIN AND R. DRUCKER
Departments of Nutrition and Microbiology, Hoffmann-La Roche, Inc., Nutley, N. J.

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When 5,6-dimethylbenzimidazole was announced as a degradation product of vitamin B_{12} (Folkers, '50), it became of interest to ascertain the effect of incorporating in the diet this compound, riboflavin and several other compounds containing the 5,6-dimethylbenzimidazole moiety, on the growth of vitamin B_{12}-deficient rats and chicks. In addition to these studies, the effect of administering these compounds in lieu of vitamin B_{12} upon the vitamin B_{12} content of the bloods, livers and kidneys of rats and chicks is described.

EXPERIMENTAL

Growth studies

Rat. Male Sprague-Dawley rats weighing 35 to 45 gm were used in these experiments, and were housed in individual cages. The vitamin B_{12} depletion diet of Register et al. ('49) was modified so that 0.01% crystalline DL-thyroxine replaced the iodinated casein. The rats were fed the diet ad libitum for a two-week depletion period, at the end of which time they were divided into groups of 15 or 20 each according to weight and placed on the assay regimen for two weeks.

[1] Roche Publication 270. A preliminary report of this work was presented before the American Society of Biological Chemists, meeting in Cleveland, Ohio, May, 1951.

In the first experiment riboflavin and various synthetic compounds shown in table 1 were assayed for growth-promoting ability. The compound 5,6-dimethylbenzimidazole, and other water-insoluble compounds, were dissolved in hydrochloric acid and adjusted to pH 4.5. Crystalline vitamin B_{12} [2] served as the standard, and all compounds were given by intraperitoneal injection three times a week. The results are shown in table 1. It is apparent that in this experiment, of the compounds tested, riboflavin gave the greatest weight response. In other experiments, 100 μg of riboflavin per day produced no weight response, whereas the weight effect observed when 5 mg of riboflavin were fed per day was no greater than that observed at the 2 mg per day level. A combination of 0.05 μg of vitamin B_{12} and 2 mg riboflavin provided no greater weight gain than that level of vitamin B_{12} alone. The compound 5,6-dimethylbenzimidazole provided a weight response at the 1 mg per day level, but at the 5 and 10 mg levels proved toxic. The compound 1,2-diamino-4,5-dimethylbenzene was without effect at the 2 mg per day level, but at the 3 mg level some growth was noted. The compound 1-(1′-D-ribityl)-5,6-dimethylbenzimidazole was only active at the 10 mg per day level. On the other hand, 2-(D-ribo-tetrahydroxybutyl)-5,6-dimethylbenzimidazole was inactive at the 1 and 10 mg per day levels.

When these experiments were completed, a paper by Emerson et al. ('50) appeared which indicated a higher order of activity for 5,6-dimethylbenzimidazole and 1,2-diamino-4,5-dimethylbenzene than we had obtained under our conditions. Accordingly, it was decided to test these compounds, substituting Emerson's diet ('49) for that used in our experiments. It was observed that when the rats were maintained on the depletion diet as described by Emerson ('49), for 4 weeks, a great many succumbed before going on test. It was subsequently found that reducing the depletion period to two weeks resulted in fewer deaths without affecting the

[2] Merck.

TABLE 1

TABLE 1

Growth-promoting ability of several synthetic compounds for rats fed vitamin B_{12}-depletion diets

SUPPLEMENT PER DAY	MODIFIED DIET OF REGISTER ET AL. ('49)		EMERSON DIET ('49)	
	Intraperitoneal administration	Oral administration	Intraperitoneal administration	Oral administration
	Average gain (gm) for two-week assay period			
0	34 ± 3[1] (20)[2]	31 ± 3 (20)	24 ± 3 (20)	12 ± 3 (20)
0.05 μg B_{12}	55 ± 2 (20)	50 ± 2 (20)	53 ± 2 (20)	45 ± 3 (20)
0.20 μg B_{12}	77 ± 2 (20)	67 ± 3 (20)	71 ± 3 (20)	60 ± 3 (20)
2 mg riboflavin	50 ± 3 (20)	50 ± 3 (20)		
1 mg 5,6-dimethylbenzimidazole	46 ± 3 (15)		34 ± 3 (20)	
2 mg 5,6-dimethylbenzimidazole		44 ± 3 (20)		30 ± 3 (20)
3.5 mg 5,6-dimethylbenzimidazole				25 ± 3 (20)
5 mg 5,6-dimethylbenzimidazole	46 (2 dead) ± 4 (15)	46 ± 3 (20)		
10 mg 5,6-dimethylbenzimidazole	all dead (15)			
2 mg 1,2-diamino-4,5-dimethylbenzene	34 ± 3 (15)	41 ± 3 (20)	30 ± 3 (20)	
3 mg 1,2-diamino-4,5-dimethylbenzene	41 ± 3 (15)			
5 mg 1,2-diamino-4,5-dimethylbenzene				34 ± 4 (20)
1 mg 1-(1′-D-ribityl)-5,6-dimethylbenzimidazole	34 ± 3 (15)			
5 mg 1-(1′-D-ribityl)-5,6-dimethylbenzimidazole		42 ± 3 (15)		
10 mg 1-(1′-D-ribityl)-5,6-dimethylbenzimidazole	47 ± 3 (15)			
0.2 mg 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole		53 ± 2 (15)		
1 mg 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole		66 ± 2 (15)		
0.2 mg 1-β-D-ribofuranosido-5,6-dimethylbenzimidazole		48 ± 3 (15)		
1 mg 1-β-D-ribofuranosido-5,6-dimethylbenzimidazole		51 ± 3 (15)		
10 mg 2-(D-ribo-tetrahydroxybutyl)-5,6-dimethylbenzimidazole		38 ± 3 (15)		
1 mg 2-(D-ribo-tetrahydroxybutyl)-5,6-dimethylbenzimidazole		34 ± 3 (15)		

[1] Standard error of mean, $\sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$.

[2] Number in parentheses represents number of animals in group.

weight response to vitamin B_{12}. Following two weeks of depletion a two-week assay period was used, and the rats were given the compounds by intraperitoneal injection three times a week. These results are shown in table 1, and parallel closely the results we obtained with the modified diet of Register et al. ('49) and similar means of administration.

Since the possibility existed that the parenteral toxicity of these compounds was inhibiting the growth response, it was decided to test the growth stimulating effects of these compounds when given orally. Solutions of the material tested were introduced into the rats' stomachs with the use of a syringe and blunted needle, and the rats were fed in this manner three times a week. In the first experiment employing oral administration, the modified diet of Register et al. ('49) was used. In addition to the compounds used in the previous experiments, 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole and 1-β-D-ribofuranosido-5,6-dimethylbenzimidazole were tested for activity. It is interesting to note that 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole was most effective under these conditions, 1 mg per day producing growth equivalent to the 0.20 μg per day level of vitamin B_{12}. The compound 1-β-D-ribofuranosido-5,6-dimethylbenzimidazole, on the other hand, was less active. The activity of these two compounds was somewhat less than was observed by Emerson et al. ('51) under their experimental conditions.

When administered orally, the activity of riboflavin and 5,6-dimethylbenzimidazole was not substantially changed, whereas 1,2-diamino-4,5-dimethylbenzene had a somewhat higher activity when given orally than when given parenterally.

In the next experiments, 5,6-dimethylbenzimidazole and 1,2-diamino-4,5-dimethylbenzene were given orally 5 times a week to rats depleted with the diet of Emerson ('49). The results are shown in table 1. Although these compounds possessed activity at the levels used, the rats did not attain maximum weight.

Chick. One-day-old, single-comb White Leghorn chicks hatched from eggs obtained from hens maintained on a vitamin B_{12}-low ration were used in these experiments. They were housed in an electrically heated brooder and fed the basal ration shown in table 2. The chicks were given this ration for three days, at the end of which time giants and runts were discarded. The remaining chicks were wing-banded and divided according to weight into groups of 20 each. The supplements were mixed directly into the ration, and the birds were maintained on the experimental diets for 4 weeks. Several of the compounds used in the rat experiments and, in addition,

TABLE 2

Composition of vitamin B_{12}-low basal chick ration [1]

CONSTITUENTS	AMOUNT	CONSTITUENTS	AMOUNT
Ground yellow corn (%)	26	Riboflavin (mg/kg)	22
Ground whole wheat (%)	20	Niacin (mg/kg)	220
Soybean meal (%)	50	Ca pantothenate (mg/kg)	110
Dicalcium phosphate (%)	2	Vitamin K (mg/kg)	22
Calcium carbonate (%)	1	$MnSO_4 \cdot 4H_2O$ (gm/kg)	2.2
Sodium chloride (iodized, %)	0.5		
Cod liver oil (%)	0.5		

[1] We are indebted to Dr. M. B. Gillis, School of Nutrition, Cornell University, for suggesting this ration.

1,2-dimethyl-4-amino-5-ribitylaminobenzene, were tested for growth-promoting ability under these conditions. The results are presented in table 3.

Of interest is the fact that riboflavin promoted growth for the chick as well as the rat. At the level fed, 1,2-diamino-4,5-dimethylbenzene had no activity. The ribityl derivative of this compound (1,2-dimethyl-4-amino-5-ribitylaminobenzene), on the other hand, did possess a growth-stimulating effect. The activity of 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole was of a lower order for the chick than for the rat.

Tissue studies

Rat. At the end of the rat experiment described above, in which 5,6-dimethylbenzimidazole and 1,2-diamino-4,5-di-

methylbenzene were fed orally 5 times per week, the animals in several of the groups were sacrificed and the livers and kidneys analyzed microbiologically for vitamin B_{12} content. Prior to killing the rats, blood samples were obtained for analysis from the tail vein. In each case 5 rats were used from each group and the various tissues were pooled. Results were calculated on the basis of micrograms per gram of wet tissue.

The tissues were blended with distilled water in a Waring Blendor and a dilution of this material was used for assay without further treatment. In addition, an aliquot portion of

TABLE 3

Growth-promoting effect of several synthetic compounds in chicks fed a vitamin B_{12}-low diet

SUPPLEMENT PER KILO RATION	WEIGHT GAIN 4 WEEKS
	gm
0	139 ± 5 [1]
10 μg B_{12}	182 ± 5
40 μg B_{12}	194 ± 6
200 mg 5,6-dimethylbenzimidazole	162 ± 7
200 mg 1,2-diamino-4,5-dimethylbenzene	130 ± 6
200 mg 1,2-dimethyl-4-amino-5-ribitylaminobenzene	157 ± 7
200 mg riboflavin	168 ± 6
100 mg 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole	165 ± 6

[1] Standard error of mean.

each sample was boiled for one-half hour in 0.2 *N* NaOH to provide a non-vitamin B_{12} stimulator blank (Hoffmann et al., '49). By employing a technique which we have developed of assaying with *Lactobacillus lactis Dorner* in the presence and absence of the cyanide ion in the medium, some evaluation of the state of vitamin B_{12} in the tissue is permitted (Cooperman et al., '51). The results are given in table 4. It is apparent that the rats fed vitamin B_{12} had larger stores of this vitamin in the liver and kidney than the rats in the other groups. It is interesting to note that the free vitamin B_{12} content in the blood of the rats in the various groups was low and that assay with cyanide did not yield increased values.

On the other hand, cyanide treatment of the kidney and liver samples produced increased amounts of apparent vitamin B_{12}. Whether this indicates a conversion of vitamin B_{12} to other forms during preparation of the samples, or an actual store of other forms of vitamin B_{12}, cannot be deduced from

TABLE 4

Vitamin B_{12} content of blood, livers and kidneys of rats fed various supplements

GROUP	L. LACTIS ASSAY		AFTER BOILING IN 0.2 N NaOH	NET B_{12} CONTENT
	No CN	CN		
		Blood mμg/ml		
1. Negative control	1.3	1.0	0.5	0.5
2. 0.2 μg B_{12} per day	1.3	1.5	0.2	1.3
3. 3.5 mg 5,6-dimethyl-benzimidazole per day	0.8	1.0	0.4	0.6
4. 5 mg 1,2-diamino-4,5-dimethylbenzene per day	0.7	0.9	0.3	0.6
		Liver mμg/gm (wet)		
1. Negative control	12	15	5	10
2. 0.2 μg B_{12} per day	22	35	5	30
3. 3.5 mg 5,6-dimethyl-benzimidazole per day	11	17	4	13
4. 5 mg 1,2-diamino-4,5-dimethylbenzene per day	12	20	6	14
		Kidney mμg/gm (wet)		
1. Negative control	28	63	12	51
2. 0.2 μg B_{12} per day	93	180	11	169
3. 3.5 mg 5,6-dimethyl-benzimidazole per day	35	80	12	68
4. 5 mg 1,2-diamino-4,5-dimethylbenzene per day	34	78	10	68

these data. These results confirm the observations of Lewis et al. ('49) that in the rat the kidney has a higher concentration of vitamin B_{12} than the liver.

Chick. When the chick growth studies were completed, the birds were killed and the kidneys and livers removed for vitamin B_{12} assay. The tissues from 5 birds from each group

were pooled. The samples were blended in a Waring Blendor and aliquots were taken for direct assay, for digestion with trypsin (Thompson et al., '50), for digestion with pancreatin (Couch and Orlando, '50), and for boiling in 0.2 *N* NaOH. An aliquot from each enzyme treatment was boiled in 0.2 *N* NaOH for one-half hour. The method of Cooperman et al. ('51), employing *Lactobacillus lactis Dorner* with a basal medium containing cyanide, was used for microbiological assay. The results are shown in table 5. Since the two enzyme treatments provided similar results, only those results obtained with pancreatin are shown. Several of the samples were also assayed with *L. leichmannii*[3] and similar results were obtained in each case.

It is evident that when the tissues were analyzed without an enzymatic digestion, the livers and kidneys of the chicks receiving vitamin B_{12} had by far the largest stores of this vitamin. Little difference was noted among the other groups. After enzymatic digestion, an interesting phenomenon was noted. In addition to the increase in values for total *L. lactis*-stimulating material, there was a marked increase in alkali-stable stimulating material. When several enzyme treated samples were assayed with *L. leichmannii,* equally high values for alkali-stable, microbiologically stimulating material were obtained. The nature of this alkali-stable, stimulating material has not been determined.

DISCUSSION

Several simple compounds containing the 5,6-dimethylbenzimidazole moiety are shown to possess a limited capacity to stimulate the growth of rats and chicks maintained on diets deficient in vitamin B_{12}. Of the compounds tested by us, 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole and riboflavin have been found to be most active.

Rats maintained on the modified diet of Register et al. ('49) gave very similar responses to the compounds used,

[3] United States Pharmacopeia Study of the Microbial Assay for Vitamin B_{12} Activity, August 15, 1950.

TABLE 5

Vitamin B_{12} content of kidneys and livers of chicks fed various supplements

SUPPLEMENT PER KILOGRAM	VITAMIN B_{12} CONTENT IN MμG/GM (WET)					
	No enzyme treatment			Pancreatin digestion		
	1. Apparent B_{12} content	2. Not destroyed by alkali	3. Net 1–2	1. Apparent B_{12} content	2. Not destroyed by alkali	3. Net 1–2
			Kidney			
1. 0	20	4	16	220	138	82
2. 40 μg B_{12}	408	12	396	630	116	514
3. 200 mg 5,6-dimethylbenzimidazole	50	14	36	225	140	85
4. 200 mg 1,2-diamino-4,5-dimethylbenzene	20	9	11	321	130	191
5. 200 mg 1,2-dimethyl-4-amino-5-ribitylaminobenzene	25	10	15	296	148	148
6. 200 mg riboflavin	12	4	8	300	85	215
7. 100 mg 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole	10	4	6	370	173	197
			Liver			
1. 0	6	1	5	162	135	27
2. 40 μg B_{12}	314	3	311	519	182	337
3. 200 mg 5,6-dimethylbenzimidazole	11	5	6	196	122	74
4. 200 mg 1,2-diamino-4,5-dimethylbenzene	10	4	6	173	100	73
5. 200 mg 1,2-dimethyl-4-amino-5-ribitylaminobenzene	11	4	7	188	100	88
6. 200 mg riboflavin	13	1	12	253	115	138
7. 100 mg 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole	15	5	10	193	119	74

as did rats maintained on the Emerson ('49) diet. There appears to be a slight advantage in the oral mode of administration versus the parenteral, but this advantage may be due to a lower oral toxicity of the compounds tested.

The growth response obtained in chicks was found to be qualitatively very similar to that observed in rats. Only 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole produced a growth response in either rats or chicks to equal that given by an adequate supplement of vitamin B_{12}. This is in contrast to the work reported by Emerson et al. ('50) on rat growth response to 5,6-dimethylbenzimidazole and 1,2-diamino-4,5-dimethylbenzene. Our results are in qualitative agreement with those of Emerson et al. ('51) with regard to 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole. Hartman et al. ('49) have noted the growth-promoting response to riboflavin of rats maintained on a vitamin B_{12}-deficient diet.

In the case of the synthetic compounds assayed, there appeared to be no consistent correlation between growth and tissue stores of vitamin B_{12}. When no enzyme treatment was employed, both the livers and kidneys contained vitamin B_{12} in an amount comparable to that of the negative controls. After enzymatic digestion there was some variation, with the chicks receiving riboflavin containing the highest content of vitamin B_{12} in the liver and kidney. However, in no case were values obtained approaching those of the chicks receiving vitamin B_{12}.

Although several of the synthetic compounds tested can promote growth in rats and chicks fed vitamin B_{12} depletion diets, these compounds do not appear to be especially effective in increasing the vitamin B_{12} content of the blood, kidney or liver. In the chick, riboflavin was the most effective of the non-B_{12}-containing substances tested in increasing the vitamin B_{12} content of the liver and kidney.

In our experiments, in both the rat and the chick, the kidney appears to a richer source of vitamin B_{12} than the liver. Couch and Orlando ('50) state that in the chick the liver appeared to be the principal organ of storage.

Either trypsin or pancreatin is suitable for obtaining increased values for the vitamin B_{12} content of chicken liver and kidney. In addition, these enzymes appear to release substances which stimulate the growth of both *L. lactis Dorner* and *L. leichmannii* but which are alkali-stable. Further experiments are necessary to determine which method of sample preparation will yield results comparable to the true biological value.

SUMMARY

1. Several synthetic compounds were tested for their growth-promoting ability in rats and chicks maintained on vitamin B_{12}-deficient diets. The compound 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole was found to be most potent. Among the other compounds possessing activity were 1-β-D-ribofuranosido-5,6-dimethylbenzimidazole, riboflavin, 1(1′-D-ribityl)-5,6-dimethylbenzimidazole, 5,6-dimethylbenzimidazole, 1,2-dimethyl-4-amino-5-ribitylaminobenzene, and 1,2-diamino-4,5-dimethylbenzene.

2. The livers and kidneys of rats and chicks fed vitamin B_{12}-low diets supplemented with a variety of compounds containing the 5,6-dimethylbenzimidazole moiety were assayed for vitamin B_{12} content. Only those animals fed vitamin B_{12} had appreciable stores of this vitamin in the kidneys and livers.

3. There appeared to be no consistent correlation between growth and tissue stores of vitamin B_{12} when the chicks were fed several synthetic compounds.

4. Digestion of tissues with pancreatin or trypsin prior to assay results in markedly elevated vitamin B_{12} assays. However, there is also an increase in microbiologically-active material not destroyed by boiling in 0.2 *N* NaOH for one-half hour.

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EFFECT OF FASTING OR PROTEIN DEPLETION UPON BODY WEIGHT, AND FAT, NITROGEN, AND CHOLINE CONTENT OF LIVERS OF CHOLINE-DEFICIENT RATS [1]

O. M. HALE AND A. E. SCHAEFER

Department of Animal Husbandry and Nutrition, Agricultural Experiment Station of the Alabama Polytechnic Institute, Auburn

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The use of the protein depletion technique for studying amino acid utilization by the rat was ably demonstrated by Frazier et al. ('47). Best ('34) reported that large doses of choline given over long periods before and during fasting prevented accumulation of liver fat. Deuel et al. ('37) and Best and Ridout ('38) showed that during fasting choline accelerated the disappearance of fat from the livers of animals in which fatty livers had been produced previously. Wong et al. ('49) studied the effect of low and large supplies of choline upon the changes in liver composition of rats subjected to severe protein depletion. There was a rapid loss of liver nitrogen while fat accumulated. They noted that the fat accumulation was only partially preventable with choline.

It is the purpose of this paper to report the results of two depletion-repletion techniques for developing choline deficiency in rats. One technique used was to subject young adult rats to fasting with and without oral choline, followed

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by refeeding with adequate protein diets with and without choline. The other technique was to subject rats to protein depletion followed by protein repletion, using diets deficient in choline, vitamin B_{12}, and folacin.

EXPERIMENTAL

Young adult rats of the Alabama Experiment Station (AES) strain, reared on a stock diet, females weighing 160 to 180 gm and males weighing 180 to 200 gm, were placed in individual cages and uniformly distributed with respect to weight, sex, and number (4 per group). Water was supplied ad libitum to all groups. The repletion diet was composed of extracted peanut meal 30,[2] extracted casein 6,[3] sucrose 39.5, minerals (undried) 4.4,[4] L-cystine 0.1, cod liver oil 1.0, and lard 19.0%. Vitamin supplements, in milligrams per kilogram of diet, were as follows: thiamine 2, riboflavin 4, pyridoxine 2, calcium pantothenate 10, niacin 20, inositol 200, alpha-tocopherol 25, alpha-tocopherol acetate 25, and 2-methyl-1,4-naphthoquinone 5. The repletion diet furnished a maximum of 0.007% choline and 0.3% methionine. For the depletion diet, the peanut meal and casein were replaced with sucrose. The control rats in the protein-depletion studies received a dietary supplement of 0.2% of choline chloride. In the fasting studies, the diet used during realimentation was the same as the protein-repletion diet. The rats receiving choline during fasting were given 1 ml of an aqueous solution, which contained 40 mg of choline chloride, daily by stomach tube.

At the end of the experimental period the animals were sacrificed by a sharp blow on the head, and bled. The liver was quickly excised, blotted free of adhering blood, and sampled immediately for moisture, fat, nitrogen and choline determinations. The liver-fat values reported represent the percentage of ether extract on a dry weight basis. Ni-

[2] Engel, R. W., J. Nutrition, *36:* 739, 1948.
[3] Salmon, W. D., J. Nutrition, *33:* 155, 1947.
[4] See footnote 3.

trogen was determined on the ground, moisture-free liver by the micro-Kjeldahl method. Choline was determined by the method of Engel ('42) with the following modifications: The sample was continuously extracted for 48 hours with boiling methanol and normal propyl alcohol was used as a wash solution for the choline reineckate. All analyses were made in duplicate on individual livers. Values reported are averages of the data obtained for 4 rats for each experimental group.

TABLE 1

Effect of fasting with and without oral choline on body weight and liver composition of rats

DAILY CHOLINE SUPPLEMENT	FASTING PERIOD	CHANGE IN BODY WT. FROM INITIAL WT.	FINAL BODY WEIGHT	LIVER WT. / BODY WT.	LIVER ANALYSIS			
					Moisture	Fat (dry basis)	Nitrogen (dry basis)	Choline (dry basis)
mg	*days*	*gm*	*gm*	*%*	*%*	*%*	*mg/gm*	*mg/gm*
0	1	—17	181	3.4	69.2	14.2	129	13.3
0	3	—30	154	3.3	67.4	23.3	120	12.0
0	5	—48	151	3.1	68.4	21.9	112	11.2
0	7	—63	132	2.8	69.4	16.5	126	11.1
0	10	—83	113	2.2	75.2	7.9		
40.0	1	—20	182	3.6	68.3	14.1	129	13.3
40.0	3	—36	157	3.0	69.4	13.5	123	12.7
40.0	5	—52	143	3.0	69.5	11.7	123	11.9
40.0	7	—60	138	3.2	70.4	10.7	127	10.4
40.0	10	—82	118	1.9	76.1	8.3		

RESULTS

Fasting

The results of subjecting rats to fasting with and without oral choline feeding are given in table 1. Forty milligrams of choline chloride given daily by stomach tube did not prolong the survival time of starved rats. Both groups survived for an average of 10 days. The rats receiving the choline chloride lost approximately the same amount of body weight during fasting as did the rats receiving no choline,

and there was only a slight difference in the liver-weight-to-body-weight ratio between the two groups.

After one to three days of fasting there was a marked increase in liver fat, from 14.2% to 23.3% (dry basis), for the rats that did not receive choline, while the liver fat of the controls gradually decreased throughout the experiment.

The oral administration of choline during fasting had no effect on liver nitrogen or choline concentration. In both groups there was a marked decrease in total liver nitrogen and liver choline as the period of fasting was increased.

TABLE 2

Influence of choline on body weight and liver composition in refed rats following 7 days of fasting

CHOLINE SUPPLEMENT	REFEEDING PERIOD	CHANGE IN BODY WT. FROM INITIAL WT.	FINAL BODY WEIGHT	LIVER WT. / BODY WT.	LIVER ANALYSIS			
					Moisture	Fat (dry basis)	Nitrogen (dry basis)	Choline (dry basis)
%	*weeks*	*gm*	*gm*	%	%	%	*mg/gm*	*mg/gm*
0	1	−3	171	6.6	54.8	68.7	39	6.0
0	2	+10	201	7.0	50.0	65.9	45	4.6
0	3	+19	193	7.6	49.0	68.9	39	3.8
0.2[1]	1	+10	201	3.9	70.7	8.5	109	13.0
0.2[1]	2	+44	261	4.0	69.1	11.1	98	10.7
0.2[1]	3	+63	287	3.0	68.3	12.8	104	9.4

[1] During the fasting period these rats had received 40 mg choline chloride daily by stomach tube.

Refeeding after fasting

The results of subjecting rats to refeeding following a 7-day fasting period with and without choline supplementation are shown in table 2. The control rats receiving 40 mg of choline chloride orally during fasting and 0.2% dietary choline chloride during the refeeding made much faster gains in body weight than did the rats fed a low-choline diet following 7 days of fasting. At the end of three weeks of refeeding, the control rats had gained three times as much weight as had the choline-deficient rats.

Maximum fatty infiltration of the liver occurred in the choline-deficient group after one week of refeeding (68.7% fat, dry basis), and remained at this high level throughout the experiment. The livers of control rats contained a normal amount of fat throughout the refeeding period.

The nitrogen content of the livers of rats fed the choline-deficient diet for one to three weeks after fasting was considerably lower than that of controls when expressed as mil-

TABLE 3

The effect of protein depletion for varying periods with and without dietary choline on body weight and liver composition of rats

CHOLINE SUPPLEMENT	DEPLETION PERIOD	CHANGE IN BODY WT. FROM INITIAL WT.	FINAL BODY WEIGHT	LIVER WT. / BODY WT.	LIVER ANALYSIS			
					Moisture	Fat (dry basis)	Nitrogen (dry basis)	Choline (dry basis)
%	*days*	*gm*	*gm*	%	%	%	*mg/gm*	*mg/gm*
0	4	—16	183	4.1	67.4	29.4	85	7.3
0	8	—31	156	4.1	66.5	22.0	81	6.4
0	12	—41	154	3.2	60.2	28.4	83	6.8
0	14	—35	174	3.6	63.2	37.7	63	5.1
0	16	—50	157	4.3	63.7	34.6	71	4.6
0	20	—53	168	4.3	50.3	57.0	62	3.3
0.2	4	—31	178	4.0	69.4	11.2	95	9.3
0.2	8	—33	161	4.4	69.3	10.9	84	8.3
0.2	12	—42	156	3.0	64.3	15.0	99	8.5
0.2	16	—47	156	3.9	68.9	13.1	89	7.9
0.2	20	—47	162	3.9	67.9	17.7	81	6.2

ligrams nitrogen per gram dry liver (39 to 45 mg as compared to 98 to 109 mg, respectively). This difference was not apparent when the nitrogen was calculated on a dry, fat-free basis.

The livers of control rats contained more than twice as much choline per gram dry liver as did the livers of choline-deficient rats.

Protein depletion

The results of subjecting rats to protein depletion for from 4 to 20 days with and without choline supplementation

are shown in table 3. The fat content of the livers of the rats on the low-choline depletion diet increased from 20.4% (dry basis) after 4 days' depletion to 57.0% after 20 days, as compared with 11.2% and 17.7%, respectively, for the controls receiving 0.2% choline chloride.

During depletion, the choline concentration of the livers showed a gradual decline in both groups. The livers of the control rats contained approximately 2 mg more choline per gram dry liver at any of the periods of depletion than did the livers of the choline-deficient rats. When the choline concentration of the liver was calculated on a dry, fat-free basis, the livers of the control group contained approximately 1 mg more choline per gram than did the livers of the choline-deficient group.

There was a gradual decrease in liver nitrogen content, from 85 to 62 mg per gram of dry liver in the choline-deficient rats, and from 95 to 81 mg in the controls, in a 20-day depletion period. When calculated on the basis of dry, fat-free liver, however, the nitrogen content remained relatively constant.

Protein repletion after depletion

The results of subjecting rats to protein repletion with and without choline supplementation following 21 days of protein depletion with and without choline supplementation are shown in table 4. The choline-deficient animals receiving a diet during repletion which supplied 21% protein barely regained in three weeks the body weight lost during depletion, whereas the controls (receiving 0.2% choline chloride) regained this loss plus an additional 80 gm. The liver fat of the choline-deficient group during the first week of repletion was about 5 times higher than the liver fat of the controls (68.4% compared to 12.8%, dry basis). The livers of the control rats contained nearly three times as much nitrogen and choline per gram of dry liver as did the livers of the choline-deficient rats, but when this value is converted

to milligrams per gram of dry, fat-free liver, there was no significant difference between the two groups.

DISCUSSION

The oral administration of choline chloride during fasting periods of from one to 10 days had no apparent beneficial effect upon body weight loss or survival. During the refeeding period, choline supplements greatly increased body weight gain either after fasting for 7 days or after protein depletion for three weeks.

TABLE 4

Influence of choline on body weight and liver composition in rats fed the repletion diet following 21 days of protein depletion

CHOLINE SUPPLEMENT	REPLETION PERIOD	CHANGE IN BODY WT. FROM INITIAL WT.	FINAL BODY WEIGHT	LIVER WT. / BODY WT.	LIVER ANALYSIS			
					Moisture	Fat (dry basis)	Nitrogen (dry basis)	Choline (dry basis)
%	*weeks*	*gm*	*gm*	%	%	%	*mg/gm*	*mg/gm*
0	1	— 28	169	6.0	49.8	68.4	35	4.2
0	2	— 38	152	6.4	48.3	68.6	37	3.8
0	3	+ 1	191	6.5	44.9	68.4	41	3.5
0.2 [1]	1	+ 9	211	3.8	69.8	12.8	104	10.2
0.2 [1]	2	+ 40	220	3.6	69.7	13.4	99	10.4
0.2 [1]	3	+ 80	261	3.6	70.8	11.6	94	10.2

[1] During protein depletion these rats received 0.2% choline chloride.

During protein depletion or fasting, the administration of choline had no apparent effect on liver size. When the animals were repleted, the choline-deficient rats had a liver-weight-to-body-weight ratio approximately twice as great as that of the controls. This may be accounted for by the extensive fatty infiltration that occurred in the livers of the choline-deficient animals. After one or three weeks of repletion following either fasting or protein depletion, the liver fat of the choline-deficient rats was approximately 6 times as great as the liver fat of the controls. Three days of fasting without oral choline produced maximum liver fat. Continued fasting beyond this time reduced liver fat below the

initial value. Choline-deficient rats subjected to protein depletion for 4 to 20 days had a gradual increase in liver fat. When the repletion diet, unsupplemented with choline, was fed after three weeks of depletion or 7 days of fasting, liver fat concentration reached a maximum after one week and did not change during two additional weeks of repletion.

Changes in the apparent concentration of nitrogen or choline in the dry liver were for the most part caused by changes in the fat content. The nitrogen and choline concentrations in the dry, fat-free liver were relatively constant.

The supply of adequate amounts of protein to animals after either fasting or protein depletion obviously is no guarantee of rapid recovery. Diets containing 20% protein and nutritionally adequate except for choline and vitamin B_{12} can actually accentuate choline deficiency. These results suggest that an adequate supply of dietary choline should be included in any therapy for starvation or protein depletion.

SUMMARY

1. Two techniques for producing choline deficiency in the rat were used. In one, the rats were subjected to fasting and refeeding, with and without choline; in the other they were subjected to protein depletion and repletion with and without choline.

2. Rats not receiving choline during fasting and refeeding or during protein depletion and repletion barely regained body weight loss after three weeks of refeeding or repletion. In contrast, rats similarly treated but receiving choline regained body weight loss and in addition gained 63 to 80 gm.

3. Extremely fatty livers (68% fat, dry weight basis) were obtained in the choline-deficient rats fed the 20% protein diet for one week following 7 days of fasting or 21 days of protein depletion. Under similar conditions, normal liver fat was maintained in rats receiving adequate dietary choline.

4. Concentration of nitrogen and choline in the livers (dry basis) of rats receiving adequate dietary choline during re-

feeding or repletion was approximately three times higher than in similarly treated rats not receiving choline. However, when liver nitrogen and choline were calculated on a dry, fat-free basis, the concentrations were not significantly different for the two groups.

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THE EFFECT OF DIETARY FAT ON THE REPRODUCTIVE PERFORMANCE AND THE MIXED FATTY ACID COMPOSITION OF FAT-DEFICIENT RATS[1]

F. A. KUMMEROW, H. P. PAN AND H. HICKMAN
Department of Food Technology, University of Illinois, Urbana

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It has been shown in previous studies (Evans et al., '34; Maeder, '37; Mackenzie et al., '39; Quackenbush et al., '42) that reproduction failure always ensues in female rats which have grown to maturity on a fat-free diet. After a prolonged gestation period and excessive vaginal bleeding, the young are born dead or die soon after birth. However, if the diet is supplemented with linoleic or arachidonic acid or natural oils containing these fatty acids, normal reproduction ensues. For example, when female rats which had grown to maturity on a fat-free diet were supplemented daily with as little as 100 mg ethyl linoleate three weeks before they were bred, the gestation period was normal and up to 83% of the young were weaned (Quackenbush et al., '42).

The role which dietary sources of essential fatty acids play in reproduction was not elucidated by fat analyses of mother and young. Quackenbush et al. ('42) stated that a remarkable constancy was noted in the iodine values and the percentages of total fat extracted from the animals which had been kept on the fat-free and from those kept on the fat-supplemented diets.

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It has recently been shown that iodine values do not necessarily reflect changes in the percentage composition of a fat produced by the feeding of dietary fats (Hite et al., '49; Kummerow et al., '49). In the present study, therefore, the percentage composition of the fats extracted from mother and young were determined with the aid of spectrophotometric analyses and the results correlated with reproduction performance. Furthermore, young were removed from representative pregnant females with the aid of Caesarean section during the latter part of the gestation period in order to determine possible causes of intra-uterine death.

EXPERIMENTAL

Preparation of animals

To minimize the pre-experimental storage of essential unsaturated fatty acids, 122 female weanling rats were fed for 17 weeks a fat-free ration which consisted of crude casein, glucose,[2] salts (Wesson, '32) and all of the known vitamins in adequate amounts (table 1). The animals were then divided into 6 groups; two groups were fed the known B complex vitamins in crystalline form, and the remainder were fed rice bran concentrate[3] fortified with choline chloride, riboflavin, *p*-aminobenzoic acid and folic acid. In addition, two groups were fed 5% corn oil and one group 5% hydrogenated fat.

At 17 weeks of age the animals weighed 172 to 186 gm. They seemed normal in appearance except for a slight scaliness of the hind paws and tail. The animals were mated with normal males. Eighteen days after sperm were found in the vaginal smear, the animals were segregated in individual cages and weighed daily. From the 18th to the 22nd day of the gestation period, a 4th of the pregnant animals which had been kept on the fat-free rations were anesthetized

[2] Cerelose.
[3] Vitab.

and the young removed from the uterus by means of Caesarean section.

Fat analyses of the carcass

Two different methods were used for carcass fat analyses; one involved a saponification and the other a solvent ex-

TABLE 1

Composition of rations

COMPONENT	FED TO GROUP NUMBER					
	1	2	3	4	5	6
	gm	gm	gm	gm	gm	gm
Casein [1]	180	180	180	180	180	180
Wesson salts	40	40	40	40	40	40
Cerelose	780	730	750	750	700	700
Corn oil	..	50	..	..	50	..
Hydrogenated fat [2]	..	..	..	..	..	50
Rice bran conc. [3]	..	..	30	30	30	30
Vitamin supplement	[4]	[4]	[5]	[6]	[5]	[5]

[1] Plus 0.33 gm cystine.

[2] Crisco, Proctor and Gamble Co., Ivorydale, Ohio.

[3] Vitab, Nopco Chemical Co., Harrison, N. J.

[4] Inositol 99.0 mg, choline chloride 308 mg, niacin 26 mg, riboflavin 1.0 mg, thiamine 3.0 mg, *p*-aminobenzoic acid 3.0 mg, folic acid 0.8 mg, calcium pantothenate 7.0 mg, pyridoxine hydrochloride 2.5 mg, and biotin 0.04 mg added per kilogram of basal ration.

[5] Choline chloride 120 mg, riboflavin 0.8 mg, *p*-aminobenzoic acid 3.0 mg, and folic acid 0.7 mg added to every 30 gm of Vitab.

[6] Cystine 2.2 gm, inositol 860 mg, choline chloride 2.0 gm, niacin 54 mg, riboflavin 8.7 mg, thiamine 0.96 mg, calcium pantothenate 3.7 mg, pyridoxine hydrochloride 1.6 mg, *p*-aminoazobenzoic acid 3.0 mg, folic acid 0.7 mg and biotin 0.015 mg added to every 30 gm of Vitab. The Vitab was increased from 3 to 6% and the concentration of vitamins added to ration 1 and 2 was doubled after the rats had been bred.

traction procedure. In the former, three representative animals and their newly born young were saponified in 30% aqueous potassium hydroxide, freed of non-saponifiable material, and the fatty acids obtained as previously described (Quackenbush and Steenbock, '42). In the latter, the intestinal tract was removed from the mature animals and

the remaining carcass extracted with Skelly-solve F. The weighed carcass was placed in a Waring Blendor and an equal weight of anhydrous sodium sulfate and Skelly-solve F added and blended for two minutes. The tissues were allowed to settle, the solvent decanted and the extraction repeated three times. The tissues were kept in contact with the solvent for at least 8 hours during the 4th extraction period.

The combined Skelly-solve F extracts were dried with sodium sulfate, transferred to a tared round bottom flask, freed from solvent under vacuum and weighed. The residue was separated into neutral fat and phospholipids by precipitation from acetone. Both the acetone-soluble or neutral fat and the acetone-insoluble or phospholipid fractions were freed from solvent under vacuum, weighed and subjected to spectrophotometric analysis as previously described (Brice et al., '45; Potter and Kummerow, '50). The young were put through a similar procedure.

RESULTS

Reproduction and lactation

A failure of normal parturition always ensued unless corn oil or hydrogenated fat was included in the diets. Labor began at term in the animals which had been kept on the fat-free diets, but parturition was accompanied by excessive hemorrhage. Parturition was not completed for two or three days, while the animals lost weight and became extremely weak and anemic. Approximately 14% of the females died before giving birth to their young; 80% of the latter were born dead, some in an advanced stage of decomposition. The dead young weighed approximately the same as normal living young. All of the young which were born alive died within 24 hours after birth. On Caesarean section, living young were found in every animal examined at the 20th and 21st days of gestation. However, dead young began to appear in the uterus after the 21st day of the gestation period.

When corn oil or hydrogenated fat was included in the diets, normal parturition was noted. The animals on the diet which contained 5% corn oil lactated normally and weaned 85% of their young. However, all of the young from the animals on the diet which contained 5% hydrogenated fat died within 72 hours after birth.

Fat analyses

The results obtained in total fat analyses indicated that the animals which had been kept on a fat-free diet were

TABLE 2

The yield of total fat extracted from mother and young, its iodine value and mixed fatty acid composition

CATEGORY OF INTEREST	MOTHER		YOUNG	
	Fat-deficient diet	Corn oil diet	Fat-deficient diet	Corn oil diet
	%	%	%	%
Yield of total fat	12.2	14.7	1.2	1.3
Iodine value	75.0	84.8	110.6	116.0
Mixed fatty acid composition of total fat				
Linoleic acid	2.6	16.2	2.7	7.6
Trienoic acid	0.0	0.0	2.4	0.0
Arachidonic acid	2.5	8.8	10.2	17.2
Oleic acid	68.3	29.4	72.2	49.6
Saturated acids	26.5	45.5	12.4	25.4

deficient in arachidonic acid and not deficient in fat per se. The animals which had been kept on the fat-free diet contained approximately the same percentage of total fat as those which had received supplements of corn oil.

The mature animals and young on the former diet contained 12.2 and 1.2%, and those on the latter diet contained 14.7 and 1.3% fat, respectively (table 2). However, the data obtained on spectrophotometric analyses indicated that the fatty acid composition of these fats differed markedly. The animals which had received corn oil contained almost 4

times more arachidonic acid than those on the fat-free diet, or 8.8 and 2.5%, respectively. This difference in arachidonic acid content was also reflected in the offspring from these animals. The young from the animals which had received corn oil contained almost twice as much arachidonic acid as those from the animals on the fat-free diet, or 17.2 and 10.2%, respectively.

TABLE 3

The percentage composition of the mixed fatty acids of the neutral fat and the phospholipid from adult female rats

GROUP	RATION	LINOLEIC	TRIENOIC	ARACHIDONIC	OLEIC ACID	SATURATED FATTY ACIDS
		%	%	%	%	%
	Neutral fat					
1	Cryst. vitamins	3.2	0.0	1.0	71.3	24.2
2	Cryst. vitamins + corn oil	6.4	0.0	1.4	75.7	16.5
3	Vitab	0.7	0.3	0.2	68.6	30.1
4	Vitab + cryst. vitamins	0.6	0.5	0.3	68.9	29.7
5	Vitab + corn oil	11.3	0.0	1.2	60.0	27.5
6	Vitab + hydrog. fat	1.6	0.3	0.5	74.2	23.4
	Phospholipid					
1	Cryst. vitamins	2.3	0.0	3.8	64.0	29.9
2	Cryst. vitamins + corn oil	13.1	0.0	3.4	39.1	44.4
3	Vitab	1.7	0.4	2.2	53.8	41.9
4	Vitab + cryst. vitamins	1.3	0.3	1.2	59.4	37.9
5	Vitab + corn oil	11.4	0.0	5.0	42.1	41.5
6	Vitab + hydrog. fat	6.5	0.0	4.9	40.7	52.1

The fat-deficient animals seemed to synthesize oleic and a trienoic acid in a vain attempt to increase the percentage of unsaturated fatty acids in the carcass fat. The mature animals and young on the fat-deficient diet contained 38.9 and 22.6% more oleic acid than those on corn oil, or 68.3 and 29.4, and 72.2 and 49.6%, respectively.

The arachidonic acid seemed to be concentrated in the phospholipid fraction in both the mature animals and young (table 3). The most striking differences were noted in the phospholipid fraction of the fat extracted from the young

(table 4). The young from the animals which had received corn oil contained from 5 to 10 times more arachidonic acid than those from the animals on the fat-free diet, and twice as much arachidonic acid as those from the animals on hydrogenated fat. It was interesting to note that the trienoic acid was not present in the phospholipid fraction of the animals which had received dietary sources of preformed fat.

TABLE 4

The percentage composition of the mixed fatty acids of the neutral fat and the phospholipid from newly born young

GROUP	RATION	LINOLEIC	TRIENOIC	ARACHIDONIC	OLEIC AND SATURATED ACIDS
		%	%	%	%
	Neutral fat				
1	Cryst. vitamins	6.4	4.7	1.1	87.8
2	Cryst. vitamins + corn oil	9.4	0.0	1.5	89.1
3	Vitab	3.2	4.4	2.0	90.4
4	Vitab + cryst. vitamins	3.4	5.0	2.7	88.9
5	Vitab + corn oil	9.5	0.0	1.9	88.6
6	Vitab + hydrog. fat	4.6	0.2	0.9	94.3
	Phospholipid				
1	Cryst. vitamins	6.4	5.1	4.7	83.8
2	Cryst. vitamins + corn oil	12.1	0.0	24.8	63.1
3	Vitab	8.2	6.5	2.1	83.2
4	Vitab + cryst. vitamins	9.6	4.7	1.9	83.8
5	Vitab + corn oil	11.9	0.0	21.9	66.2
6	Vitab + hydrog. fat	11.0	0.0	11.6	77.4

DISCUSSION

The present results indicate that a dietary source of unsaturated fat, such as corn oil, is necessary for normal reproduction and lactation. Furthermore, a commercially available hydrogenated fat did not furnish a sufficient amount of unsaturated fat to meet the requirements for normal lactation. The analytical data presented in this paper, and the results of Quackenbush et al. ('42) indicate that the linoleic acid in corn oil, or linoleic acid per se, is

the limiting factor involved in normal reproduction. The linoleic acid served as a source of arachidonic acid; the latter could not be synthesized without it. The newly synthesized arachidonic acid seemed to be concentrated in the phospholipid fraction and must have played a vital role in the reproduction process. In this connection, it has been noted that the degree of unsaturation of the lipids in human blood gradually increases until term (Muhlbock, '37). This increase in unsaturation may have been due to an increased synthesis of arachidonic acid. The spectrophotometric data indicate that animals which did not receive a dietary source of arachidonic acid synthesized a trienoic acid.

Nunn and MacLean ('38) isolated a simliar trienoic acid, from the fat of rats which had been kept on a fat-free diet. These workers found this trienoic acid to be a C_{20} acid, and named it dihydroarachidonic acid. It is interesting to note that an acid of this type has not been found in the phospholipid fraction of rat (Kummerow et al., '49) or poultry fat, although it has been found in the neutral rat fraction (Chu and Kummerow, '50) and in the fat extracted from heart tissue (Rieckehoff et al., '49). The dihydroarachidonic acid could represent a vain effort of the body to synthesize the essential arachidonic acid.

Whether the young which contained the trienoic acid were normal could not be determined. They were alive in the uterus of the mother, indicating that this fatty acid did not interfere with some vital function during intra-uterine life, nor did it seem to interfere in the metabolic functions of the mature animals.

The difficulty noted during parturition could have been due to the poor muscle tone of the uterine wall and may have involved a fundamental change in the mixed fatty acid composition of the lipoproteins in the muscle fiber of the uterus. Szent-Gyorgyi ('47) has pointed out that myosin which has been repeatedly recrystallized still contains 3% lipid material. Part of the latter seemed to be an alcohol-soluble

cephalin capable of functioning as a powerful thrombokinase. The excessive loss of blood in our female rats which had been kept on the fat-free diet may therefore have been due to a failure of the animals to build lipoproteins which contained the cephalin necessary for proper thrombokinase activity.

No excessive hemorrhage was noted in the animals which had received hydrogenated fat. Therefore, the hydrogenated fat seemed to contain enough linoleic acid to furnish a sufficient amount of this fatty acid to synthesize the necessary cephalin. However, linoleic acid was not present in sufficient quantities to satisfy the requirements for normal lactation.

The B complex vitamins did not seem to be involved as adding extra vitamins and increasing the rice bran concentrate from 3 to 6% did not influence reproduction or lactation.

SUMMARY

Female rats which had grown to maturity on a fat-free diet, and bred, gave birth to young which were born dead or died soon after birth. When this diet was supplemented with 5% hydrogenated fat, the animals gave birth to living young which did not live more than 72 hours, while those fed 5% corn oil weaned 85% of their young.

Total fat analyses of representative female rats and their newly born young indicated that the animals which had been kept on the fat-free diet were deficient in arachidonic acid and not deficient in fat per se. The largest differences occurred in the phospholipid fraction of the fat extracted from the young. The phospholipids of the young from animals which had received corn oil contained from 5 to 10, and those from animals on hydrogenated fat two, times more arachidonic acid than those from rats on the fat-free diet.

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THE MEASUREMENT OF FAT ABSORPTION AND DISTRIBUTION USING ELEOSTEARIC ACID[1]

JAMES F. MEAD, DOROTHY L. FILLERUP, ARTHUR B. DECKER AND LESLIE R. BENNETT

School of Medicine, University of California at Los Angeles

TWO FIGURES

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A number of studies have been carried out with respect to the absorption, distribution and storage of fat in the animal body, using various methods of detection of the fed material. These methods have in general suffered from inaccuracy and from the time-consuming techniques involved, particularly when applied to human studies.

One method which seems to avoid many of the difficulties of the others was introduced by Miller, Barnes, Kass and Burr ('39) and consists of the feeding of a lipide containing fatty acids having conjugated double bonds, which enable it to be determined spectrophotometrically with facility and accuracy. This group of workers applied the method with considerable success to problems of fat absorption and transport, using the methyl esters of the conjugated acids of corn oil as substrate (Barnes, Miller and Burr, '41a, b, c). Reiser has also employed the method, using 10, 12-octadecadienoic acid as the tracer material (Reiser and Bryson, '51).

In these laboratories a similar method has been used to determine the rate of fatty ester absorption in mice (Mead,

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Bennett, Decker and Schoenberg, '51) and the effect of x-irradiation on this rate (Mead, Decker and Bennett, '51). The tracer used was methyl 9, 11-octadecadienoate, which was dissolved to the extent of 10% in methyl oleate.

A preliminary application of the technique was concerned with a comparison of the absorption rate of methyl oleate and vitamin A. It was thought, since vitamin A is not hydrolyzed to a water-soluble compound and is apparently absorbed entirely by way of the lymphatics (Woytkiw and Esselbaugh, '51; Drummond, Bell and Palmer, '35) that valuable information could be gained by such a comparison, showing first whether vitamin A could be used as a tracer for fat absorption, and second, whether any further light could be thrown on the mechanism of absorption of unhydrolyzed lipides as postulated by Frazer.[2] The results of this experiment are reported below.

In a search for an adequate tracer for use in clinical studies, the spectrophotometric method using a fatty acid with conjugated double bonds seemed applicable. Preliminary tests revealed that a lipide-soluble substance present in serum shows strong end-absorption of ultra-violet light starting at about 250 mμ, thus interfering with the determination of the conjugated diene (absorption maximum = 231 mμ).

However, knowledge that conjugated trienes exhibit intense absorption maxima at wave lengths greater than 250 mμ led to the investigation of eleostearic (9, 11, 13-octadecatrienoic) acid; small amounts of this substance in serum proved to be determinable easily and with great accuracy.

Miller and Burr ('37) first studied the distribution of the trienoic acids of tung oil in rats. Using one animal at each period, they studied the appearance and changes of the spectrophotometrically measurable acids in the blood, liver, kidney, brain, skin, and adipose tissue at 24, 48 and 72 hours. They reported a rapid conversion of triene to diene in all

[2] For discussions of Frazer's and Verzar's theories of fat absorption see the appropriate chapters of the Archives des Sciences Physiologiques, Vol. 11, No. 1–4, Service des Publications du C.N.R.S., 43 rue d'Ulm, Paris (5e), 1948.

organs, followed by a gradual decline in the amount of both acids.

Reiser ('51) recently studied the appearance of conjugated acids in the eggs of hens fed tung oil, and found similar changes occurring.

In view of the incomplete data on distribution of fatty acids in the various organs, the opportunity to conduct such a study simultaneously with studies on absorption was welcomed. The experiments reported below, therefore, will be concerned mainly with the rate of absorption of methyl oleate in mice as measured by eleostearate, the absorption and distribution of this acid in the organs of the rat, and evaluation of it as a tracer for fat absorption in animals and humans.

EXPERIMENTAL

Materials and methods

The mice used in the experiments were CF_1 strain raised in the project animal colony and weighing 34 ± 2 gm. The rats were Wistar strain females weighing 185 ± 15 gm. The preparation of methyl 9, 11-octadecadienoate has been described previously (Mead, Bennett, Decker and Schoenberg, '51). β-Eleostearic acid [3] was prepared from tung oil [4] by a revision of the method of Thomas and Thompson ('34). Methyl β-eleostearate was prepared from the acid with methyl alcoholic hydrochloric acid. It had a melting point of 0.1 to 0.4° and $E^{1\%}_{1\ cm}$ 1920 (in iso-octane) at 268 mμ.

For the mouse toxicity tests [5] a total of three massive doses of methyl eleostearate (50 mg per animal) was given to the mice within a 7-day period. The animals suffered no ill effects and showed no symptoms of any sort attributable to the treatment. Longer term experiments in which animals on a fat-

[3] The β-acid was used because of its higher melting point and greater stability with respect to conversion to other isomers.

[4] Tung oil was obtained from the Progressive Varnish Works, Los Angeles.

[5] Miller, Barnes, Kass and Burr ('39) reported that tung oil is "poorly tolerated" in the rat.

free diet received daily 10-mg supplements of the ester also failed to reveal any untoward effects.

The mouse absorption studies were carried out in the manner described previously for methyl oleate (Mead, Decker and Bennett, '51). The mixture fed consisted of methyl oleate containing 10% methyl 9, 11-octadecadienoate and 5% vitamin A acetate [6] or methyl β-eleostearate. The amounts fed varied between 37 and 105 mg and the times studied were from 15 to 90 minutes for the eleostearate, or 60 minutes for the vitamin A study. The iso-octane extracts of the stomach and intestinal washings were diluted to known volumes and their absorption of light was measured at 326 mμ (for vitamin A), at 268 mμ (for triene) and at 231 mμ (for diene). The optical densities found at 231 mμ were transformed into milligrams of ester by means of a suitable factor, while similar calculations were made for vitamin A or triene.

It was found that the peaks at 268 and 326 mμ suffered a slight decrease because of non-metabolic oxidation which unavoidably occurred during manipulation of the material. A correction factor based on the height of these peaks in the stomach was therefore applied to the calculation of the amounts in the intestines.

For study of the distribution of eleostearate in the rat, animals were fed about 250 mg of a 1:1 mixture of methyl oleate and methyl β-eleostearate. After the required time had elapsed, the animals were sacrificed and the necessary tissues were excised and set aside for extraction. The tissues studied were: stomach, small intestine, large intestine, blood, heart, adrenals, spleen, liver, kidneys and depot fat. Each organ was homogenized with 5 to 25 ml of 96% ethanol and the mixture was then either exhaustively extracted with iso-octane or was shaken thoroughly with a known volume of that solvent from which an aliquot was used for spectrophotometry. The iso-octane solutions were dried over magnesium sulfate and their absorption spectra studied from 300

[6] We are indebted to the Hoffmann-La Roche Company for a generous gift of crystalline vitamin A acetate.

to 220 mμ.[7] Important points measured were the peaks at 268 mμ (for triene) and 233 mμ (for diene). From the densities of these peaks were subtracted blanks found by carrying out identical procedures on animals which had received no oil.

Application of these background corrections was simplified by the observation that, for a given tissue, extinction coefficients at 233 and 268 mμ were proportional to that at 300 mμ, at which wave length both of the tracer materials are essentially transparent. Thus measurement of the absortion of a tissue extract at 233, 268 and 300 mμ suffices to assay its diene and triene content with fair accuracy. A total of 18 animals was used for the study, with 4 being used for the hour and two-hour points and two for the later periods.

For determination of the relationship between fat absorption and appearance in the blood, the same techniques were employed, but only the blood, small intestine, colon and stomach were studied. To obtain data for this study some shorter intervals post-administration were used. Four animals were used for the 30-minute period, and three additional for the 60-minute period.

The human subject was given 6 gm of methyl eleostearate in 0.5-gm capsules before breakfast. Five-milliliter samples of blood were taken at one-half, one, one and one-half, two, 4, 7 and 24 hours post-administration. The samples were extracted with iso-octane and the spectra taken as described above. Blanks were taken immediately after administration and were subtracted from the results.

RESULTS

The results of the determination of the relative rate of absorption of methyl oleate and vitamin A are shown in table 1. Pertinent information found in the table is as follows:

1. The amount of the mixture absorbed by the fasted animal (15 hours) is about half that absorbed by the

[7] Five-millimeter cells with fused quartz end windows were used throughout the study. Complete graphs were recorded on the Cary recording spectrophotometer.

fed animal; this was noted before (Mead, Bennett, Decker and Schoenberg, '51) in experiments in which vitamin A was not used.

2. In the fed animal the amount absorbed as calculated from the vitamin A is about 10% lower than that calculated from methyl octadecadienoate. In the fasted animal this figure is about 25% lower.

TABLE 1

One-hour absorption of vitamin A and methyl oleate in the mouse

CONDITION OF ANIMALS	AMT. ADM'D.	AMOUNT FOUND (MG) IN THE G.I. TRACT AS MEASURED BY METHYL OCTADECADIENOATE (UPPER FIGURE) OR VITAMIN A (LOWER FIGURE)			AMOUNT ABSORBED (MG), AS MEASURED BY METHYL OCTADECADIENOATE (UPPER FIGURE) OR VITAMIN A (LOWER FIGURE)	DIFF.
		Stomach	Small intestine	Large intestine		
	mg					
Normal	65.4	11.5	10.9 17.4	5.6 9.1	37.4 33.6	3.8
Normal	73.9	14.9	8.4 15.4	0 0	50.6 43.6	7.0
Normal	61.6	4.4	0 0	17.4 22.4	39.8 34.8	5.0
Fasted	56.6	3.9	35.4 42.0	0 0	17.3 10.7	6.6
Fasted	61.9	25.2	17.7 19.5	0 0	19.0 17.2	1.8
Fasted	68.4	19.1	31.6 36.1	0 0	17.7 13.2	4.5
Fasted	61.3	14.5	16.1 24.4	0 0	30.7 22.4	8.3

Data on the absorption of methyl oleate as measured by methyl 9, 11-octadecadienoate and methyl β-eleostearate are listed in table 2. Pertinent information derived from the table is:

1. The rate of absorption of the ester mixture is about twice that of the ester mixture in the absence of methyl

β-eleostearate. In previous experiments (Mead, Decker and Bennett, '51) the average amounts absorbed were shown to be 24 ± 4 mg at 0.5 hr., 31 ± 8 mg at 1 hr., and 42 ± 8 mg at 1.5 hr.

TABLE 2

The absorption of the ester mixture by mice

HOURS	AMT. ADM'D.	AMOUNTS RECOVERED (MG) AS MEASURED BY METHYL OCTADECADIENOATE (UPPER FIGURE) OR METHYL ELEOSTEARATE (LOWER FIGURE)			AMOUNTS ABSORBED (MG) AS MEASURED BY METHYL OCTADECADIENOATE (UPPER FIGURE) OR METHYL ELEOSTEARATE (LOWER FIGURE)
		Stomach	Small intestine	Large intestine	
	mg				
0.25	37.5	14.8	0	0	20.7
			2.0	0	18.7
	61.6	29.4	1.2	0	31.0
			1.3	0	30.9
0.5	55.5	18.3	0	0	37.2
			0	0	37.2
	72.5	9.5	2.3	0	60.7
			2.1	0	60.9
	52.3	9.6	0.6	0	42.1
			1.1	0	41.6
	78.8	13.8	9.6		55.4
			8.2	0	56.8
1	50.2	18.3	0	0	31.9
			0	0	31.9
	95.0	33.6	0	2.1	59.3
			0.3	1.1	60.0
	104.6	12.7	1.1	5.7	85.1
			1.9	9.3	80.7
			0	0	65.9
1.5	67.6	1.7	0	0	65.9

2. The amount absorbed is approximately the same when calculated from both diene and triene tracers, indicating that methyl β-eleostearate, unlike vitamin A, is absorbed at the same rate as the methyl oleate. This determination was complicated by the fact that, because of the high absorption rate of the mixture, it

was difficult to recover any from the intestine. This made comparison of absorption rates difficult, since measurements had to be made of the relative amounts of the two esters remaining after absorption.

In the study of the distribution of β-eleostearate in the rat, total recovery was first determined. Recovery was high during the first hour and dropped rapidly during the later periods. Whether this disappearance represents complete breakdown of the acid or merely its transformation to a substance transparent at 265 mμ is uncertain.

The distribution of the recovered eleostearate (and its metabolic products) may be seen in table 3, in which the amounts of diene and triene found in the various organs (expressed as percentages of the amounts of β-eleostearate administered) are shown for the various periods post-administration.

That triene is converted into diene is abundantly evident from the fact that, following administration of the former, absorption characteristic of the latter appears in the extracts of every organ examined. The standard used for calculating the amounts of diene was 9^t, 11^t-octadecadienoic acid, with $E^{1\%}_{1\,cm} = 1100$. Since this represents only one of the possible conjugated diene isomers, there is some uncertainty in these calculations. Another source of error stems from the fact that in some organs, especially liver and depot fat, high backgrounds had to be taken into consideration at 230 mμ. However, the relative amounts of diene in each organ are probably correct.

The sites of transformation of triene to diene are revealed by the early appearance of maximum diene concentration. In liver, kidney and depot fat this peak occurs at three hours, whereas for all other organs it occurs later than in the blood; it seems probable, therefore, that diene is transported to these latter organs by the blood.

In figure 1 is shown graphically the total absorption of eleostearate by the rat as compared with the appearance of the triene in the blood. It can readily be seen that the two

TABLE 3

Per cent recovery of β-eicostearate from the various tissues and organs

NO. ANIMALS USED	TIMES POST-ADMINISTRATION	% OF DOSE ABSORBED	ADRENAL		BLOOD		COLON		FAT		HEART		INTESTINE		KIDNEY		LIVER		SPLEEN		STOMACH	
			Tri.	Die.	Tri.	Die.	Tri.	Die.	Tri.	Die.	Tri.	Die.	Tri.	Die.	Tri.	Die.	Tri.	Die.	Tri.	Die.	Tri.	Die.
	hrs.																					
4	½	39.66	..	..	0.19	0.04	1.14	0.02	..	..	..	..	6.32	0.60	..	..	..	..	..	..	48.18	4.08
7	1	47.84	0.01	0.01	0.31	0.15	0.17	0.02	0.07	1.04	0.03	0.04	10.55	0.55	0.01	0.03	0.43	0.57	0.02	0.01	40.71	0.16
4	2	53.05	0.01	0.01	0.33	0.08	0.04	0.19	0.02	0.60	0.02	0.01	12.10	0	0.02	0.04	0.18	0.76	..	..	34.52	0.10
2	3	74.43	0.03	0.06	0.14	0.06	4.53	3.30	0.19	1.18	0.04	0.05	11.64	1.21	0.05	0.09	0.30	0.96	0.04	0.02	4.16	0.73
2	4	80.73	0.01	0.15	0.03	0.11	4.00	0.78	0.23	1.70	0.02	0.02	1.07	0.29	0.02	0.09	0.14	0.94	0	0.03	11.63	1.50
2	8	98.40	0.07	0.03	0.02	0.10	0.14	1.00	0.11	0.43	0.02	0.03	0.07	0.14	0.02	0.08	0.03	0.52	0.01	0.04	0.09	0.16
2	12	98.64	0	0.07	0	0.05	0.42	0.56	0.64	..	0.01	0.30	0.03	0.30	0	0.12	0	0.43	0	0.06	0.04	0.01
2	24	98.99	0	0.04	0	0.03	0.08	0.24	0.23	1.52	0.02	0.02	0.10	0.44	0.02	0.11	0.04	0.23	0	0.05	0.07	0.08

are related, since the points of maximum absorption rate and maximum concentration in the blood occur at approximately the same time.

The results of the human study are shown in figure 2. In this graph are plotted the actual amounts (extrapolated to total blood volume) of both diene and triene present in the

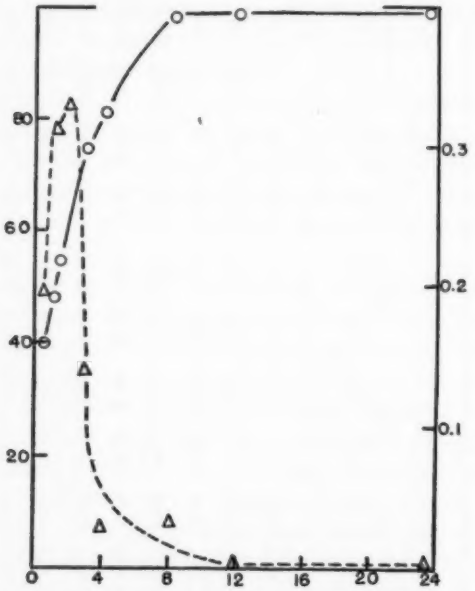

Fig. 1 Absorption of β-eleostearate by the rat. Total absorption (circles, left ordinate) and appearance in the blood (triangles, right ordinate) expressed as percentages of the dose fed.

blood at various periods following administration of β-eleostearate. It can be seen that the triene curve is very similar to that found in the rat, auguring well for its successful use as a means of calculating the absorption rate and total fat absorption in the human. The diene curve, which has its peak somewhat later than that of triene, is also seen to resemble the curve obtained for the rat. It is also of interest to note

that a quantitative measure of the post-absorptive lipemia found in the human can now be obtained, and that the amount of blood lipide for the rat in the same state is of a different order of magnitude. This phenomenon has been demonstrated qualitatively for some time and has recently been put on a quantitative basis in the latter case by Bergstrom, Borgstrom and Rottenberg ('51) but has not been investigated mechanistically.

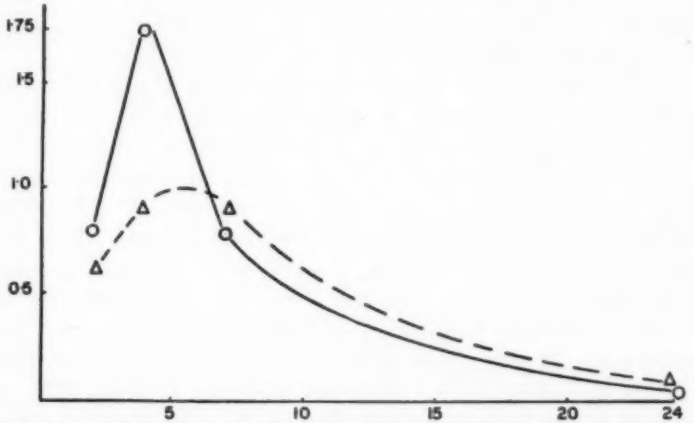

Fig. 2 The amounts (in grams) of triene (circles) and diene (triangles) present in the total blood of a human subject at various times (in hours) following administration of 6 gm of methyl, β-eleostearate.

DISCUSSION

On the basis of the data presented above several facts can be seen clearly. First, from the absorption data obtained with mice it is evident that vitamin A is not an accurate tracer for methyl oleate absorption. Indeed, if the theoretical considerations propounded below are valid, vitamin A will always be absorbed at a lower rate than fat, particularly in those cases in which fat absorption itself is poor. This fact was shown in a qualitative way by Gray, Morgareidge and Cawley ('40), who found that in the rat, fat is absorbed more rapidly than vitamin A.

In a previous communication (Mead, Decker and Bennett, '51) it was shown that mixtures of fatty esters are absorbed as a whole, and that a small amount of methyl octadecadienoate serves as an accurate tracer for the absorption of methyl oleate. It now seems probable that methyl β-eleostearate is absorbed at the same rate as the total ester mixture fed. It is possible that this fact has some bearing on current theories of fat absorption.[8] Indeed, it is difficult to see why esters with such different physical properties as oleate and β-eleostearate should be absorbed at the same rate unless they are absorbed simultaneously by the same mechanism.

One explanation for the greater absorption of methyl oleate as compared with vitamin A is that the former has the possibility of being converted to a water-soluble substance by hydrolysis and interaction with bile salts, whereas this possibility apparently does not exist for vitamin A (Woytkiw and Esselbaugh, '51; Drummond, Bell and Palmer, '35). According to Frazer's theories, a portion of the acid formed by hydrolysis may be diverted from the main pathway of absorption via the lymphatics and absorbed directly into the portal vein. If this explanation is accepted, then the portion of ester absorbed by this alternate pathway is about 10% in the case of the fed animal and 25% in the case of the starved animal.

Our earlier experiments (Mead, Bennett, Decker and Schoenberg, '51) indicated that little emulsification occurs in the intestines of fasted animals unless some glyceride is added to the ester mixture. It can be seen that the absorption of vitamin A would suffer more from this poor emulsification than would that of the ester, because of the possible availability of the alternate pathway in the case of the latter. Other interpretations of the data can, of course, be made.

Second, it is apparent that the 5% of eleostearate included in the mixture increased the rate of absorption of the whole mixture about two-fold; this noteworthy effect of eleostearate is being investigated further.

[8] See footnote 2, page 500.

Miller and Burr ('37) and Reiser ('51) have noted the transformation of triene to diene in the animal body. This interesting change may be brought about by those enzyme systems responsible for interconversion of the essential fatty acids (Holman, '51), and is being investigated both *in vitro* and *in vivo* in these laboratories. Whether the change is a simple hydrogenation (1,2, 1,4 or 1,6) or whether it involves some more complex alteration of the conjugated double bond system is not yet known.

It is not possible on the basis of the present distribution studies to decide whether β-eleostearate follows a distribution pattern similar to that of the essential fatty acids or to that of the saturated fats and oleate. The elaidate data of Kohl ('38a, b) are inconclusive from this standpoint since elaidic (like eleostearic) is not one of the normal dietary acids. Preliminary conclusions are that eleostearate and the diene formed from it are treated differently from the essential fatty acids, since the former disappear quite rapidly from the tissues while the latter are known to be retained for long periods. It is possible that data from experiments using radioactive carbon may clarify this question.

Data obtained on the rate of absorption in the rat as related to the appearance of eleostearate in the blood indicate that this substance may be useful as a tracer in studies of fat absorption in the human. The preliminary experiment described above indicated that valuable information may be gained from such a study. First, the rate and extent of fat absorption may be calculated with fair accuracy from the blood data,[9] thus permitting an evaluation of the kinetics of fat absorption. Second, the clearance of eleostearate from the blood can be measured with great ease, yielding data which may serve, for example, as a clue to the pathologic disposition of lipides such as occurs in atherosclerosis (Becker, Meyer and Necheles, '49). Finally, the appearance and disappearance of the diene may have some significance which will be brought to light in later studies.

[9] This subject is the basis of a current investigation in this laboratory.

SUMMARY

The absorption of methyl oleate by mice can be accurately measured using methyl β-eleostearate as a tracer, whereas vitamin A absorption is slower than that of the esters.

The eleostearate has also been used in tracing fat absorption in the rat by measuring both the amount disappearing from the intestine and the amount appearing in the blood. Distribution studies were carried out with rats showing the appearance and disappearance of the eleostearate in the various organs.

In one human study, blood levels of the tracer were followed, and indicated that fat absorption and blood clearance could readily be studied using small amounts of tracer and small blood samples.

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TOTAL PLASMA PROTEIN AND PLASMA FIBRINOGEN IN ASCORBIC ACID DEFICIENCY AND IN SCURVY IN THE MONKEY[1]

R. J. SALMON AND C. D. MAY

Department of Pediatrics, College of Medical Sciences, University of Minnesota, Minneapolis

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While performing bone marrow biopsies during a study of anemia in scorbutic monkeys (May et al., '51), it was noted that the bone marrow coagulated very rapidly. Analysis of the plasma for fibrinogen at such times showed it to be elevated. Search of the literature revealed a difference of opinion as to whether the plasma fibrinogen is changed in ascorbic acid deficiency. Ohata ('32) reported that the plasma fibrinogen was lowered after symptoms of scurvy had developed. He used a refractometric method for the determination of fibrinogen. Ham and Curtis ('38) found normal plasma fibrinogen in one case of scurvy which was uncomplicated by fever. They are of the opinion that, in humans, rise in plasma fibrinogen is an indication of severe tissue injury and is not influenced at all by the state of nutrition in regard to ascorbic acid. Sullivan, Gangstad and Link ('44), working with guinea pigs, found that after two weeks on a scorbutogenic diet there was a pronounced rise in plasma fibrinogen. Control animals on the same diet, and also receiving adequate supplements of *l*-ascorbic acid, showed no rise, while animals receiving inadequate ascorbic acid showed intermediate amounts.

As we had a considerable number of scorbutic monkeys under observation for various studies, we felt it would be

[1] This investigation was conducted with the aid of grants and materials from Wyeth, Inc., M. and R. Laboratories and the Eli Lilly Company.

profitable to gather more data on the relationship between ascorbic acid deficiency and the level of fibrinogen in the plasma. Because a disturbance in the metabolism of folic acid develops in the scorbutic monkey, resulting in a megaloblastic anemia (May et al., '50, '51), we sought to distinguish between the effects of ascorbic acid deficiency and those of the concomitant deficiency of folic acid. The effect of various forms of therapy on the abnormal level of plasma fibrinogen in scurvy was also ascertained. An attempt was made to distinguish the role of ascorbic acid deficiency from that of the hemorrhage which is a feature of scurvy. Also, because of the supposed relation of ascorbic acid to adrenal function, fibrinogen levels were determined before and after adrenalectomy in a monkey which was first provided with adequate ascorbic acid in the diet.

EXPERIMENTAL

Monkeys were fed various milk diets as described in other contributions from this laboratory (May et al., '50, '51). The nutritional data and information on the hematological manifestations of the animals will be found in those reports. The numbers assigned to the animals in this report are the same as in former papers. The intake of biologically adequate protein from milk was from 3 to 5 gm per kilogram of body weight per day.

Monkeys used as "controls" were fed these milk diets with adequate ascorbic acid for the same period of time as the animals not given ascorbic acid. The animals were classified as "ascorbic acid-deficient" after they had been without ascorbic acid in their diet for 30 days. They were classed as "scorbutic (normoblastic)" when signs of scurvy (periorbital hemorrhages, swollen joints and bleeding gums) had developed but the marrow was still normoblastic. After about two weeks of scurvy the marrow usually became megaloblastic and then the animals were designated as "scorbutic (megaloblastic)."

Blood for the determination of plasma protein and fibrinogen was drawn from the femoral vein without the use of a tourniquet. Clotting was prevented by a dried mixture of potassium and ammonium oxalates (0.8 gm potassium oxalate and 1.2 gm ammonium oxalate per 100 ml of solution. One milliliter was evaporated to dryness in a tube for 10 ml of blood). Total plasma proteins were determined by digestion in a micro-Kjeldahl apparatus and titration of liberated ammonia. Fibrinogen was determined by the method of Cullen and Van Slyke ('20).

TABLE 1

Total plasma protein and plasma fibrinogen of monkeys on milk diets

EXPERIMENTAL GROUPS [1]	TOTAL PROTEIN		FIBRINOGEN	
	Mean	Standard deviation	Mean	Standard deviation
	gm/100 ml		*mg/100 ml*	
Controls	8.5 (21)[2]	1.4	341 (21)	70
Ascorbic acid-deficient	9.3 (7)	1.1	414 (7)	105
Scorbutic (normoblastic)	8.4 (11)	1.5	549 (15)	95
Scorbutic (megaloblastic)	7.2 (10)	1.8	545 (10)	118

[1] Defined in text.

[2] Figure in parenthesis is number of determinations, each on a different animal.

RESULTS AND DISCUSSION

The decline in the level of total plasma protein as scurvy became progressively more severe (table 1) is probably a reflection of the marked deterioration in general nutrition and cannot be readily attributed to a specific effect of ascorbic acid deficiency. In a study of the effects of chronic scurvy in monkeys, Fraser ('42) did not find a change in total serum protein, but rather a pronounced change in the albumin-globulin ratio. The values for the total protein in our "controls" are somewhat higher than those he found in his controls. This may be due to differences in quality or quantity of protein in the diets or to the ages of the animals.

It may be seen from the results in table 1 that plasma fibrinogen began to rise during the ascorbic acid-deficient

period and became considerably elevated in the scorbutic periods. The advent of folic acid deficiency (as revealed by the appearance of a megaloblastic pattern in the marrow) did not cause further increase in the level of fibrinogen in the plasma.

The effects of various treatments upon the elevated plasma fibrinogen in scurvy are shown in table 2. The fibrinogen levels returned to values found in the control animals when the scurvy was alleviated by ascorbic acid therapy. Treatment with folic acid (PGA) or folinic acid,[2] which may be less efficiently produced in scurvy (Nichol and Welch, '50), did not restore the fibrinogen to the control levels, although the marrow did become normal. Neither vitamin B_{12} nor vitamin B_{12} and PGA combined were able to reduce the elevated plasma fibrinogen levels in scurvy to the control levels.

We had an opportunity to compare the effect on the plasma fibrinogen in scurvy of *l*-ascorbic acid and a chemically related compound, *d*-glucoascorbic acid, which we found to have no effect on the symptoms or histological lesions of scurvy. Two monkeys, numbers 82 and 91, were handled identically throughout their course and when in the "scorbutic (megaloblastic)" state were treated with *d*-glucoascorbic acid and *l*-ascorbic acid, respectively. At the end of treatment, in the animal receiving ascorbic acid the plasma fibrinogen was found to be normal, the scurvy was healing and the marrow had become normoblastic. On the contrary, at the end of treatment with glucoascorbic acid, the plasma fibrinogen remained elevated, microscopic examination revealed no evidence of healing of the scurvy and the marrow remained megaloblastic. This indicates that the effect of *l*-ascorbic acid is specific.

Ham and Curtis ('38) emphasized that plasma fibrinogen is a sensitive index of liver function. Foster and Whipple ('21a) and Schultz, Nicholas and Schaeffer ('25) showed that while extensive liver damage resulted in lowering of plasma

[2] Supplied through the generosity of Dr. Edward Campbell of the Eli Lilly Research Laboratories, where the folinic acid was synthesized.

TABLE 2

Effects of treatment on plasma protein and plasma fibrinogen of scorbutic monkeys

MONKEY NO.[1]	TREATMENT	TOTAL PROTEIN		FIBRINOGEN		MARROW AFTER TREATMENT
		Before	After	Before	After	
	days	*gm/100 ml*		*mg/100 ml*		
9	*l*-Ascorbic acid (38)	8.8	11.0	745	319	Normoblastic
33	*l*-Ascorbic acid (288)	6.4	7.2	542	377	Normoblastic
129	Vitamin B_{12} prophylactically (60)	..	7.2	...	503	Megaloblastic
14	PGA prophylactically (122)	10.3	8.8	411	540	Normoblastic
51	Folinic acid (15)	7.9	5.9	473	409	Normoblastic
12	Vitamin B_{12} (20)	..	7.4	...	514	Megaloblastic
	PGA and ascorbic acid (61)	7.4	8.2	514	343	Normoblastic
37	Vitamin B_{12} and PGA prophylactically (93)	..	..	...	631	Normoblastic
82	*l*-Ascorbic acid (12)	..	..	...	315	Normoblastic
91	*d*-Glucoascorbic acid (18)	..	..	...	484	Megaloblastic

[1] Numbers refer to animals in previous reports (May et al., '50, '51) containing detailed nutritional and hematological data.

fibrinogen, slight injury to that organ caused fibrinogen to rise. Irish and Jacques ('45) similarly reported that small doses of dicumarol stimulate fibrinogen production, while larger doses depress it. The question might be raised of whether elevation in plasma fibrinogen in scurvy reflects a slight degree of damage in the liver such as could be associated with increased plasma fibrinogen. This does not seem to be the case for two reasons: firstly, histological examination of the livers of these experimental animals did not reveal any significant degree of damage to the liver; secondly, more convincing evidence that liver function was normal was obtained in other studies performed with these same animals (Proehl and May, '52). It was found that during the scorbutic periods fecal excretion of urobilinogen was increased because of the extravasation of blood into the tissues and its subsequent destruction. In spite of this increased load of urobilinogen requiring excretion, the urinary urobilinogen remained normal, indicating adequate liver function.

Another possible explanation for the rise in plasma fibrinogen in scurvy is that acute loss of blood into the various body cavities and extravasation of blood into the tissues may excite an increased production of plasma fibrinogen. In order to test this hypothesis, blood was removed from a normal monkey in an amount estimated to be one-half its blood volume, by successive aspirations over a period of three days. The hemoglobin fell from 12.3 to 5.1 gm per 100 ml. This degree of anemia corresponds to the more extreme degree encountered in the scorbutic monkeys. On the third day, 8 ml of the monkey's blood were injected into its muscles to simulate the conditions which exist in scurvy. The results of determination of total plasma protein and plasma fibrinogen in this monkey are shown in table 3. There was a slight rise in plasma fibrinogen after considerable loss of blood and the injection of blood intramuscularly, but not nearly so great a rise as occurred in the scorbutic animals. These results are in agreement with those obtained by Foster and Whipple

('21b) following exsanguination of a dog. Hemorrhage alone is not a satisfactory explanation for the considerable elevation of fibrinogen in scurvy.

Thus it seems probable that ascorbic acid plays a role in the production of plasma fibrinogen separate from any effects of impaired liver function or hemorrhage. Because of the association of ascorbic acid with the function of the adrenal cortex, and the work of Henriques et al. ('50a, b) suggesting that the adrenal cortex may play a role in the synthesis of

TABLE 3

Effects on the plasma protein and fibrinogen of exsanguination and intramuscular injection of blood

(Control monkey no. 4E, 2.32 kg in weight; cows' milk diet plus ascorbic acid)

DAY OF EXPERIMENT	TREATMENT	HEMOGLOBIN	TOTAL PLASMA PROTEIN	PLASMA FIBRINOGEN
		gm/100 ml	*gm/100 ml*	*mg/100 ml*
1	50 ml of blood withdrawn	12.3	7.8	291
2	50 ml of blood withdrawn	8.4	7.6	268
3	20 ml of blood withdrawn, 8 ml own blood injected intramuscularly	6.5	6.5	276
4	8 ml of blood withdrawn	5.4	7.8	331
5	8 ml of blood withdrawn	5.1	7.1	398
8		6.5	7.3	365

fibrinogen, we decided to determine the effects of adrenalectomy. In a normal monkey (no. 52) adequately supplied with ascorbic acid, before adrenalectomy the total plasma protein was 7.36 gm per 100 ml and the plasma fibrinogen was 396 mg per 100 ml. One hundred sixteen days after complete bilateral adrenalectomy its total plasma protein was 6.5 gm per 100 ml and the plasma fibrinogen was 368 mg per 100 ml, while the animal, continuing to receive ascorbic acid, was maintained in fair condition without hormone therapy by the addition to the milk diet of sodium bicarbonate and sodium chloride. Later, when this adrenalectomized monkey

was rendered scorbutic, the plasma fibrinogen became elevated to 549 mg per 100 ml.

It is well known that infection can cause a marked elevation of plasma fibrinogen. The animals in these experiments were carefully observed at all times and no signs of infection were detected. Gross and microscopic examination of the animals which were killed did not reveal any evidences of respiratory or generalized infection. Not infrequently there was a mild colitis and sometimes well-localized areas of inflammatory reaction surrounding worms imbedded in the wall of the large intestine. Some of the scorbutic animals showed no evidence of infection on microscopic examination of the tissues and yet the alterations in their plasma fibrinogen were no different from those of the remainder of the scorbutic animals. It cannot be denied that minor degrees of infection may frequently have been present and have made some contribution to elevation of the plasma fibrinogen, but it is certain that infection did not play either a consistent or a major role in the manifestations shown by these animals.

CONCLUSIONS

1. Total plasma protein did not appear to be altered in scurvy more than would be easily attributable to malnutrition.
2. The plasma fibrinogen is considerably elevated in scurvy. Hemorrhage and minor degrees of infection may contribute to this elevation, but do not appear to account for it entirely.
3. Secondary deficiencies of folic acid, folinic acid or vitamin B_{12} cannot be held responsible.
4. Adrenalectomy did not affect the level of plasma fibrinogen and did not prevent its rise when the monkey was made scorbutic. Thus it seems unlikely that ascorbic acid deficiency influenced the level of plasma fibrinogen through any effect upon adrenal function.
5. It is obviously difficult to determine whether ascorbic acid plays a direct role in determining the level of protein or fibrinogen in the plasma once scurvy develops because of the complicated state of affairs which then exists.

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THE EFFECT OF PROCESSING ON THE ENZYMATIC LIBERATION OF LYSINE AND ARGININE FROM SOYBEAN OIL MEAL

D. R. CLANDININ AND A. R. ROBBLEE
Poultry Division, University of Alberta, Edmonton, Alberta, Canada

THREE FIGURES

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Methods used in processing soybeans have been shown to affect markedly the nutritive value of the resulting meals. Clandinin, Cravens, Elvehjem and Halpin ('47) showed that heating a sample of solvent-extracted raw soybean flakes in the autoclave for 4 minutes at 15 pounds' steam pressure resulted in the production of a meal of high nutritive value as measured by chick growth. When heating was continued beyond 20 minutes there was a gradual decrease in the nutritive value of the meals.

In a study of the release of essential amino acids by enzymatic hydrolysis from properly-heated and over-heated soybean oil meals, Riesen, Clandinin, Elvehjem and Cravens ('47) reported that the decreased nutritive value of the over-heated meal was associated with a decreased liberation of essential amino acids. The results suggested that *in vitro* enzymatic hydrolysis values may constitute an index of the relative nutritive value of different meals.

The present study was initiated to determine whether or not this technique might be used to evaluate the nutritive value of soybean oil meals processed under varying conditions.

EXPERIMENTAL

Two series of soybean oil meals were prepared by processing solvent-extracted raw soybean flakes[1] in an autoclave. Series I was prepared by heating samples of the flakes at 15 pounds' steam pressure for zero, two, 4, 7½, 15, 30, 60, 120, 240 and 480 minutes, and series II by heating samples of the flakes at 4 pounds' steam pressure for zero, 7½, 15, 30, 45, 60, 120 and 240 minutes. The technique employed in connection with the preparation of the meals was the same as that used by Clandinin et al. ('47).

The procedures followed for the preparation of enzymatic hydrolysates of the meals and for the determination of lysine and arginine were the same as those employed by Clandinin ('49).

Alpha-amino-nitrogen determinations on the enzymatic hydrolysates of the meals were conducted by the nitrous acid micro-method of Van Slyke ('15).

The basal ration used in the chick growth studies was the same as the one used previously by Clandinin et al. ('47).

RESULTS AND DISCUSSION

The amount of liberation of lysine and arginine by enzymatic hydrolysis from the soybean oil meals in series I and II is shown in figure 1. The results indicate that maximum liberation of lysine and arginine in series I (15 lb. steam pressure) occurred at 15 to 30 minutes of processing. In the case of series II (4 lb. steam pressure), maximum liberation was obtained at 60 minutes of processing. Heating for longer periods of time in both series resulted in a decreased liberation of these amino acids. It is interesting to note that the trends of liberation of the amino acids followed in general the growth patterns reported by Clandinin et al. ('47, '48) for meals processed under similar conditions.

However, it should be noted that there was a marked difference in the level of liberation of the amino acids from the two

[1] Supplied by the Archer-Daniels-Midland Co., Minneapolis, Minn.

series of meals. In the case of the two amino acids studied, the release was greatest from the meals processed at 15 lb. steam pressure. In the light of these findings, and since meals of optimum and equal nutritive value may be produced at either steam pressure (Clandinin et al., '48), it would appear that use of *in vitro* enzymatic release of amino acids as a measure of the nutritive value of soybean meals processed at different steam pressures does not constitute a reliable index of the nutritive value of the meals. This view is further

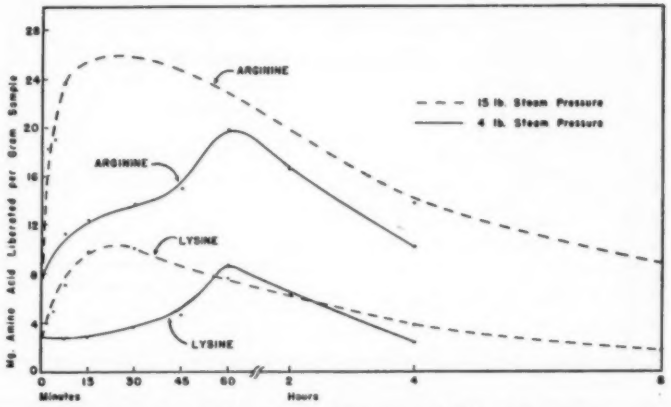

Fig. 1 Lysine and arginine liberated by enzymatic hydrolysis from soybean oil meals processed at 15 pounds and 4 pounds of steam pressure for varying periods of time.

substantiated by the fact that an equivalent release of amino acids may be obtained from meals on either side of the peaks of liberation, although very different growth results may be obtained by feeding these meals. For example, the amount of lysine and arginine released from a meal heated for 4 minutes at 15 lb. steam pressure is about the same as from a meal processed under similar conditions for two hours. However, it has been shown that a two-hour meal has a considerably lower nutritive value than a 4-minute meal (Clandinin et al., '47).

The results of the alpha-amino-nitrogen determinations on the two series of meals are shown in figure 2. It will be noted that while the alpha-amino-nitrogen values in each series followed in general the growth patterns (referred to above) on meals processed under similar conditions, the curve showing the release of alpha-amino-nitrogen of the series processed at 4 lb. pressure is considerably below the plane of the curve representing the 15 lb. steam-pressure meals. Hence, it would also appear that alpha-amino-nitrogen values would not con-

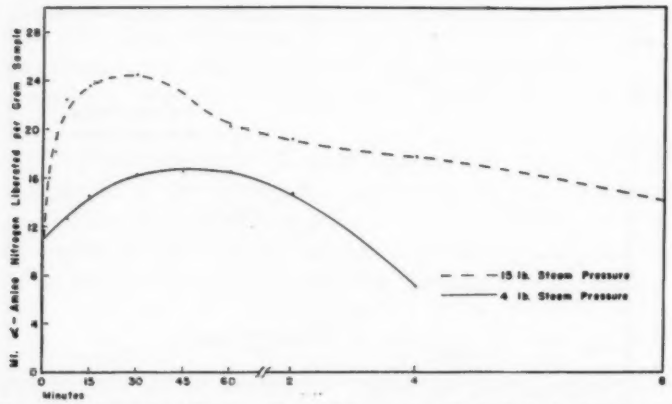

Fig. 2 Alpha-amino-nitrogen liberated by enzymatic hydrolysis from soybean oil meals processed at 15 pounds and 4 pounds of steam pressure for varying periods of time.

stitute a reliable measure of the relative nutritive value of soybean oil meals processed at different autoclave temperatures.

Since meals (4 minutes at 15 lb. steam pressure and 45 minutes at 4 lb. steam pressure) which were previously found to promote maximum growth as measured by both rate of growth technique (Clandinin et al., '47) and by 4-week feeding trials (Clandinin et al., '48) were noted to yield considerably less lysine, arginine and alpha-amino-nitrogen than meals processed for longer periods of time, the authors were led to wonder whether all the trypsin inhibitor in the meals

which promoted maximum growth was destroyed (as reported by Riesen et al., '47). However, upon re-determining the active enzyme concentration of enzyme-substrate mixtures involving each of the meals in series I and series II by the method of Riesen et al. ('47) and plotting the values obtained (fig. 3), the authors believe that the lower liberation of amino acid and alpha-amino-nitrogen from the superior meals was not the result of the presence of trypsin inhibitor, but rather the result of insufficient heat being applied to the flakes to

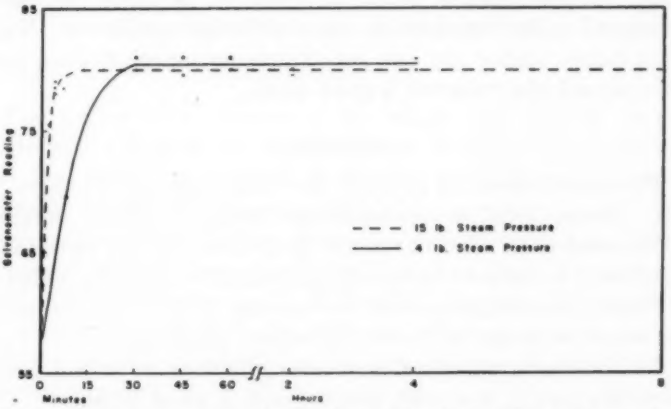

Fig. 3 Active enzyme concentration (proportionate to galvanometer reading; method of Riesen et al., '47) of enzyme-substrate mixtures involving soybean oil meals processed at 15 pounds and 4 pounds of steam pressure for varying periods of time.

modify the protein in such a way as to permit maximum *in vitro* enzymatic digestion.

While it is clear, from the growth data presented by Clandinin et al. ('47, '48) and the amino acid and alpha-amino-nitrogen release data presented in figures 1 and 2, that once the peaks of enzymatic release are reached, meals of progressively decreasing quality are produced, it is also apparent that good meals (flakes processed 4 to 30 minutes at 15 lb. steam pressure and 30 to 60 minutes at 4 lb. steam pressure) may yield appreciably different quantities of amino acids

and alpha-amino-nitrogen as the result of *in vitro* enzymatic digestion. Further, meals which promote good growth (flakes processed 4 minutes at 15 lb. steam pressure or 45 minutes at 4 lb. steam pressure) may yield amino acid or alpha-amino-nitrogen values similar to those of meals which support depressed growth rates (flakes processed two hours at 15 lb. steam pressure). Hence it would appear that amino acid or alpha-amina-nitrogen values obtained subsequent to *in vitro* enzymatic hydrolysis do not necessarily constitute a reliable index of the relative nutritive value of soybean oil meals processed under the same or under different conditions. Maximum values under any one set of processing conditions, however, would characterize a good meal.

SUMMARY

The data presented support the following conclusions:

1. Amino acid or alpha-amino-nitrogen values obtained subsequent to *in vitro* enzymatic hydrolysis do not necessarily constitute a reliable index of the relative nutritive value of soybean oil meals processed for varying periods of time under the same or under different autoclave conditions.

2. Maximum values at any one autoclave processing temperature would, however, characterize a good meal.

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FACTORS AFFECTING THE PERCENTAGE OF CERTAIN PROTEINS IN EGG WHITE[1]

FRANK A. CSONKA AND MERRIAM A. JONES

Bureau of Human Nutrition and Home Economics and Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C.

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INTRODUCTION

The amino acid content of the whole egg as well as egg white and egg yolk can be altered by feeding the laying hen diets in which the protein is varied in kind and quantity (Csonka et al., '47; Csonka, '50). In the present article we report electrophoretic studies on egg whites obtained from eggs laid by hens fed a high-protein-casein, high-protein-soybean, or low-protein basal diet. The purpose of these experiments is to observe the influence of the genetical lineage and the dietary protein on the percentages of the several egg-white proteins in different breeds.

Longsworth, Cannan and MacInnes ('40) observed slight variations in the percentages of ovalbumin and conalbumin in the white of chicken eggs. The feed of the laying hens as an influencing factor, however, was not considered. In a recent publication by Forsythe and Foster ('50), electrophoretic studies (at pH 7.8) made on eggs collected from groups of chickens of different breeds indicated small, but statistically significant, differences in egg-white composition. These changes were attributed strictly to genetic differences among the laying hens, without reference to the hens' diet.

[1] This is the third article in a series dealing with the influence of dietary protein. A preliminary report was presented before the Federation of American Societies for Experimental Biology, 1951, annual metting, held at Cleveland, Ohio. Fed. Proc., Part 1, *10:* 176. (1951).

EXPERIMENTAL AND DISCUSSION

Leghorn-Rhode Island Red Crosses and two types of standard-bred pullets, Rhode Island Red and Leghorn, in their first year laying season were used in these experiments.[2] Three types of diets, similar in composition to those reported previously, were fed: (1) a low-protein basal diet in which corn meal furnished the main protein source; (2) a high-protein diet in which 20 parts of commercial casein were substituted for 20 parts of corn meal in the basal diet; and (3) a high-protein diet in which 50 parts of soybean meal were substituted for 50 parts of corn meal.

Three weeks after changing the hens' diet the eggs were collected. Electrophoretic analyses were made on whites of individual eggs and on composite samples from 5 egg whites of eggs from 5 hens. For the preparation of the individual egg-white sample the white of one egg was separated as completely as possible from the yolk, and the yolk was washed thoroughly with a 2% NaCl solution. The white and washings were then combined and diluted to 200 ml with a 2% NaCl solution. In the composite egg-white sample, 5 egg whites were treated similarly but finally diluted to 1 l. After being gently revolved at intervals for several hours, the liquid was stored overnight at 4°C. Part of this solution was then centrifuged to remove the white precipitate (mainly mucin), and two 50-ml aliquots of the supernatant solution were dialysed separately for 24 hours at 4°C. against each of three changes of acetate buffer, pH 4.5, ionic strength 0.1.

The electrophoretic analyses, using a double length single section cell, were run in a Tiselius apparatus at 1.6°C. The procedure described by Longsworth et al. ('40), including their scanning technique, was followed. The constant current device employed was that described by Levine and McCarthy ('47). Electrophoresis was continued for three hours

[2] The cooperation rendered by the Poultry Section of the Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, is gratefully acknowledged by the authors.

(10,800 sec.) under a potential gradient of 7 volts per centimeter. Pattern measurements were made on 3 × enlargements. When resolution of components was incomplete, areas were delineated by a vertical line from the minimum between peaks. The sections, each representing a certain type of protein, were cut from the enlarged patterns and weighed. From the descending patterns percentages of each were then calculated with respect to the sum of the weights.

Tables 1 and 2 present data illustrating the influence which the dietary protein has on the percentage of the several egg-white proteins of chicken eggs.

TABLE 1

Electrophoretic analyses of individual egg whites, from eggs laid by two pullets, Rhode Island Red and Leghorn crosses

HEN NO.	DIET	OVOMUCOID	OVALBUMIN	CONALBUMIN	TOTAL GLOBULINS
		%	%	%	%
1	Low-protein	15.7	58.9	15.1	10.3
1	High-protein-soybean	14.3	59.5	15.2	11.0
2	Low-protein	17.5	60.3	12.8	9.5
2	High-protein-soybean	15.6	60.8	12.4	11.3

Table 1 shows the results of electrophoretic analysis of individual egg whites. The pullets were on a low-protein diet first, followed by a high-protein-soybean diet. The dietary change caused the most noticeable difference in the ovomucoid and the total globulin fraction percentages.

The electrophoretic experiments shown in table 2 were carried out on two aliquots taken from the same composite sample of 5 egg whites, from eggs laid by 5 Rhode Island Red pullets. Here again we observe, although a different kind of high-protein diet was used, changes in the ovomucoid and total gloublins brought about by varying the hens' diet. Individual determinations are given beside the averages so

that one may observe the accuracy of the electrophoretic method of analysis from the replicates.

The egg-white-protein compositions reported by Longsworth et al. ('40) at pH 3.92 to 5.32 are very similar to the averages we found in the albumin of eggs laid by hens fed the high-protein-soybean diet. The composition of their hens' diet was not given. Forsythe and Foster ('50) likewise did not describe the hens' diet but, since the eggs were obtained

TABLE 2

Electrophoretic analyses of egg whites from eggs laid by Rhode Island Red pullets fed low- and high-protein diets

ANALYSIS NO.[1]	DIET	OVOMUCOID	OVALBUMIN	CONALBUMIN	TOTAL GLOBULINS
		%	%	%	%
1	Low-protein	13.6	62.8	12.3	11.3
2	Low-protein	14.2	59.7	13.2	12.8
Average	Low-protein	13.9	61.3	12.8	12.0
3	High-protein-casein	12.6	60.7	14.7	12.0
4	High-protein-casein	13.1	58.0	14.1	14.8
Average	High-protein-casein	12.9	59.4	14.4	13.4

[1] Numbers 1 and 2 represent analyses of aliquots for a composite mixture of 5 egg whites from eggs laid by 5 different pullets; numbers 3 and 4 represent a second composite prepared similarly.

from two institutions, it is probable that the compositions of the feeds used were not identical. The differences they observed in the percentages of the egg-white proteins may, therefore, be attributed to the combined influences of two variables: (1) inheritable characteristics; and (2) the diet. In our work these two variables could be observed separately.

The mean percentages for Rhode Island Red and Leghorn breeds in table 3 were based on the composite samples described above, while the mean percentages of the crosses were based on one egg-white analysis for each of three

eggs from three different hens. A dietary effect on the proportion of the various egg-white proteins was more noticeable in the Rhode Island Red than in the other breeds (table 3). In this breed the ovomucoid and ovalbumin content was higher in the egg white representing the low-protein dietary regimen. The high-protein-casein dietary regimen produced higher conalbumin and globulin-2 percentages than the low-protein feed. The foregoing differences, although not great, were always present.

The genetic differences among the three groups of chickens (table 3) may be responsible for certain changes in the egg-white-protein ratios presented in the tables. Differences observed between the means of the individual egg-white proteins, when the three groups of chickens were fed the same type of diet, may well be attributable to genetics. The tendency shown is that the average percentages of ovomucoid and conalbumin (with one exception) on the two diets were greater in the eggs of the crosses (table 3), while those of the total globulins were greater in the eggs of the standard-breds. These differences were similar under both the low-protein and high-protein dietary regimens.

To test the genetical significance of the differences between means of protein fractions in egg whites (table 3), 95% confidence intervals were computed.[3] The cross-bred fed on either high- or low-protein diets showed significantly higher values of ovomucoid than the Rhode Island Red. When diets were combined in the analysis, both cross-bred and Leghorn were significantly higher than the Rhode Island Red. When standard-breds were combined, the cross-bred values were significantly higher than those of the standard-bred. The confidence intervals for conalbumin and ovalbumin overlap between breeds whether dietary protein levels are considered separately or combined. For total globulin, the Leghorn, on low- and high-protein diets, had significantly higher values than the cross-bred on a low-protein diet. Combined stand-

[3] The authors wish to thank Mr. A. Parks for making the statistical analyses presented here.

TABLE 3

Mean percentages[1] *of the proteins in egg whites calculated from replicate electrophoretic analyses as influenced by the diet and genetic lineage of the hen*

BREED	HENS' DIET	OVOMUCOID	OVALBUMIN	GLOB. 3	GLOB. 2	CONALB.	GLOB. 1	TOTAL GLOB.
Rhode Island Red	Low-protein	13.9 ± 0.4	61.3 ± 0.8	3.9	4.3	12.8 ± 0.4	3.9	12.0 ± 0.6
	High-protein-casein	12.9 ± 0.4	59.4 ± 0.8	4.5	5.2	14.4 ± 0.4	3.8	13.4 ± 0.6
Leghorn	Low-protein	15.1 ± 0.4	58.7 ± 0.8	4.2	4.3	13.1 ± 0.4	4.6	13.1 ± 0.6
	High-protein-casein	14.7 ± 0.4	59.1 ± 0.8	3.6	3.5	13.2 ± 0.4	6.5	13.0 ± 0.6
Leghorn X Rhode Island Red Crosses	Low-protein	16.0 ± 0.4	60.0 ± 0.7	4.3	3.3	14.2 ± 0.3	2.5	10.0 ± 0.5
	High-protein-casein	15.6 ± 0.4	59.1 ± 0.7	5.0	3.5	14.3 ± 0.3	2.4	10.9 ± 0.5

[1] The standard error of the mean was based on error mean square from analysis of variance.

ard-bred values were higher than the cross for the high-protein as well as for the low-protein diet. When diets were combined in the analysis, both of the standard breeds, individually and together, showed significantly higher values than the cross-bred. Globulin 1 was identical in behavior with total globulin, except that the the Leghorn was also significantly higher in this respect than the cross-bred on a high-protein diet.

SUMMARY

The percentages of the proteins in chicken egg white, as determined electrophoretically, are influenced by the nature and quality of the dietary protein of the laying hen, as well as by genetical differences in lineage.

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THE EFFECTS OF HEAT ON THE NUTRITIVE VALUE OF PROTEIN HYDROLYSATES WITH DEXTROSE

LACY R. OVERBY AND DOUGLAS V. FROST
Abbott Laboratories, North Chicago, Illinois

THREE FIGURES

(Received for publication November 26, 1951)

The reaction between amino acids and reducing sugars (Maillard, '12, '16) has been studied generally by measurement of browning. Although the simultaneous development of color and fluorescence in the browning reaction has been known since 1945 (Olcott and Dutton, '45), it is only recently that definite relationships between the two characteristics have been suggested. Friedman and Kline ('50a), and Patton and Chism ('51) have pointed out that the colored and fluorescing compounds are not identical and that the fluorogens may be precursors of the brown pigments (melanoidins).

Parenteral hydrolysates with dextrose show wide variations in growth promotion in animal feeding experiments (Cannon, '50). Hydrolysates with dextrose may undergo severe loss in nutritive value during preparation or upon storage. It seems likely that in most cases this nutritional deterioration is due to the loss of individual amino acids through reaction with dextrose.

No official standards for determining the nutritive value of protein hydrolysates have been established. The rat repletion assay, however, offers a convenient procedure for estimation of the over-all nutritive value (Cannon, '48; Frost, '50). The purpose of the present studies was to analyze fibrin hydrolysates by the rat repletion assay, and to cor-

relate changes in nutritive value with color and fluorescence changes, loss of amino nitrogen, and loss of specific amino acids.

METHODS OF STUDY

Five per cent fibrin hydrolysate solutions, as prepared for commercial use in one-liter bottles, were fortified with glucose and heated in an autoclave at 10 pounds pressure (115°C.). The pH of the fibrin hydrolysate-dextrose solutions studied was 5.5 to 5.65 initially. After 80 minutes of autoclaving the increase in acidity was insignificant, as represented by a change of only 0.1 pH point. Periodically, usually at 10- or 20-minute intervals, samples were removed for analysis. The properties of the solutions were determined as follows:

1. Color. The optical density at 385 mμ was measured in a Beckman Model "DU" quartz spectrophotometer. In cases which required dilutions to be made in order for the density to be read, all samples in the series were diluted to one part to 10.

2. Fluorescence. A Coleman photoelectric fluorophotometer with PC-1 (input) and B-1 (output) filters was used. Results on the undiluted samples were read as per cent of a quinine sulfate standard containing 0.0005 mg per milliliter in 0.1 N sulfuric acid.

3. Amino acids. The individual amino acids were determined microbiologically. Tryptophan was determined colorimetrically by the method of Graham et al. ('47).

4. Amino nitrogen. The Van Slyke procedure was carried out with three-minute deaminization in the Fischer deaminizer described by Koch ('24).

5. Nutritive amino acid value was determined by the rat repletion method (Frost and Sandy, '48). Assays were carried out for 5 days at 0.2 gm N per rat per day according to the routine method used in production control (Frost and Sandy, '49) or at the more critical level of 0.12 gm N in a 12-day assay.

RESULTS

Color and fluorescence changes in fibrin hydrolysate-glucose solutions

The rates of color formation in 5% fibrin hydrolysate solutions are summarized in figure 1. For reference, curve I represents browning of the hydrolysate in the absence of glucose. There is little or no color development when the

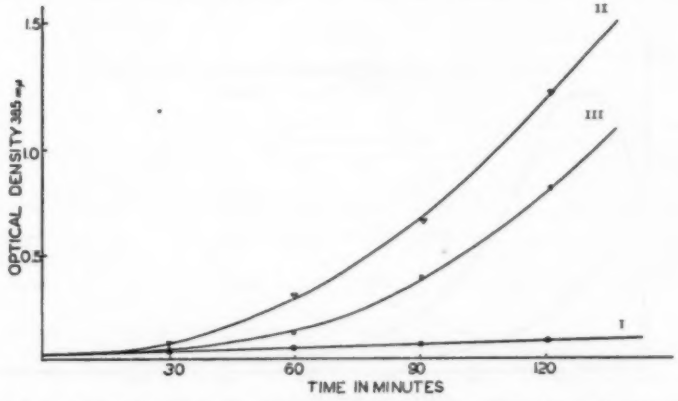

Fig. 1 Rate of browning of fibrin hydrolysate (5%) and glucose (5%) solutions autoclaved at 115°C.

● Fibrin hydrolysate,

△ fibrin hydrolysate + glucose,

□ fibrin hydrolysate + glucose + $Na_2S_2O_4$.

amino acids are heated alone. However, in the presence of 5% glucose (curve II), there is a very rapid color development following a short induction period, indicating an autocatalytic type of reaction. The color inhibitory action of sulfites is illustrated in curve III. All of the inorganic sulfites tested effectively lengthened the induction period. In the presence of sodium hydrosulfite (0.02%), there was little browning for about 30 minutes, and the slopes of curves II and III differed up to about 100 minutes. The curves fi-

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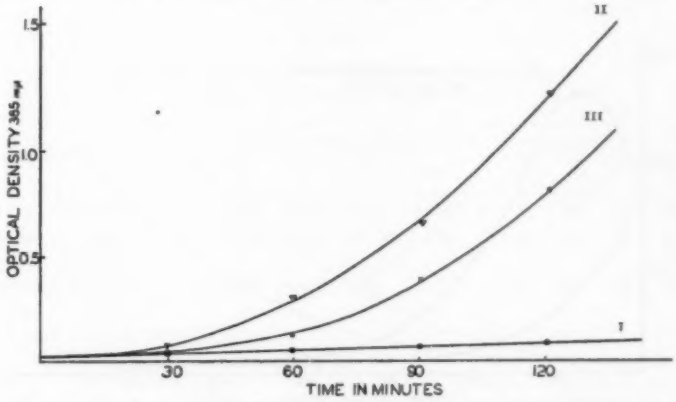

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nally became parallel, indicating that the inhibitory properties of the sulfite were expended.

Figure 2 shows the relationship between color and fluorescence development. Fluorescence curves are superimposed over the corresponding color curves shown in figure 1. Freshly prepared fibrin hydrolysate showed an initial fluorescence corresponding to a value of about 175 on the quinine sulfate scale. Autoclaving in the absence of glucose resulted in very little change in the degree of fluorescence.

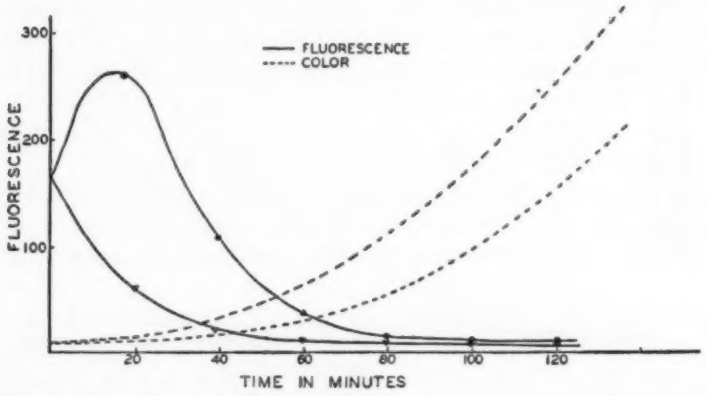

Fig. 2 Relation of fluorescence to color development in fibrin hydrolysate (5%) and glucose (5%) solutions autoclaved at 115°C.
△ Without sulfite,
● with 0.02% $Na_2S_2O_4$.

However, heating with 5% glucose gave a rapid decrease in the fluorescence, as is shown in the lower curve. With 0.02% sodium hydrosulfite there was a rapid increase in fluorescence, a maximum was reached, and this was followed by a characteristic decrease to a constant low value.

Color and fluorescence changes in glycine-glucose solutions

Parallel experiments with 0.25 molar solutions of glycine and glucose showed color and fluorescence changes similar

to those observed with hydrolysate-glucose solutions. The unheated glycine-glucose solutions showed little fluorescence, but there was a rapid development of fluorescence before marked browning occurred. In the absence of sulfite the fluorescence disappeared rapidly. With sulfite, color was inhibited, but the fluorescence maximum was increased and its duration extended. Data illustrating the relation between color and fluorescence in glycine-glucose solutions are given in table 1.

TABLE 1

Color and fluorescence development in glycine-glucose solutions autoclaved at 121°C.

MINUTES OF AUTOCLAVING	OPTICAL DENSITY 385 mμ				FLUORESCENCE [1]			
	0	30	60	120	0	30	60	120
Glycine (0.25 M)	0.022	0.022	0.027	0.036	16	20	24	28
Glucose (0.25 M)	0.029	0.013	0.018	0.036	12	12	12	20
Glycine (0.25 M) + glucose (0.25 M)	0.017	0.446	1.28	.. [2]	16	120	40	16
Glycine (0.25 M) + glucose (0.25 M) + $NaHSO_3$ (0.05%)	0.004	0.112	0.180	0.319	16	72	144	172

[1] As per cent of a standard quinine sulfate solution containing 0.0005 mg per milliliter in 0.1 N sulfuric acid.

[2] Solution was opaque.

Relation of nutritive value to color, fluorescence, and amino nitrogen changes

Heated hydrolysates gave no significant decrease in nutritive response up to an optical density of about 1.5 at 385 mμ. Beyond this point rapid nutritional deterioration occurred. Although the lighter colored solutions generally showed the higher fluorescence, no constant correlation of nutritive value and fluorescence was obtained. Solutions originally well-balanced with respect to amino acids showed nutritional stability during the period of maximum fluorescence.

Figure 3 summarizes the results, correlating the changes in nutritive value in relation to color, fluorescence and amino nitrogen in a progressively heat-browned solution. There was a slow, gradual loss of amino nitrogen, followed by a more rapid decrease. The results suggest that two phases occur in the reaction between amino acids and reducing sugars. First there is a period of little nutritive loss corresponding to the color induction period, the build-up of fluores-

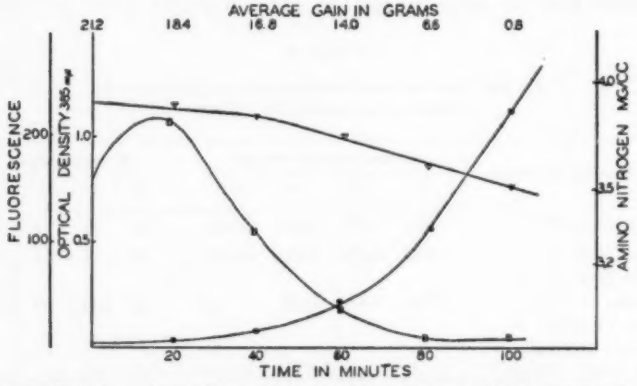

Fig. 3 Relation of nutritive value to amino nitrogen, fluorescence and color of fibrin hydrolysate (5%) and glucose (5%) solution autoclaved at 115°C.

△ Amino nitrogen,

□ fluorescence as per cent of a standard quinine sulfate solution containing 0.0005 mg per milliliter in 0.1 N sulfuric acid,

● optical density at 385 mμ.

cing intermediates, and faint signs of the disappearance of amino nitrogen. Following this there is a rapid nutritional deterioration, corresponding to rapid color formation, decay of the fluorescence, and a more rapid amino nitrogen loss. The 60- to 80- minute period in figure 3 indicates the period during which the rapid nutritional loss began. During this period the amino nitrogen decreased by about 2.5%. Such a rapid loss of nutritive value could be accounted for if one or more of the essential amino acids were removed, giving a nutritionally unbalanced mixture.

Relation of nutritive value to loss of specific amino acids

Progressively browned hydrolysates were analyzed microbiologically to determine the relative rates of disappearance of the individual amino acids. The results of one such series are recorded in table 2. These values represent the actual milligrams of each amino acid ingested per rat per day in the 5-day rat repletion assay. For comparison, the minimum daily requirements which support a rapid rate of recovery

TABLE 2

Microbiological assay of heat-darkened fibrin hydrolysate-glucose solutions

MINUTES AT 115°C.	MG OF AMINO ACID PER 200 MG TOTAL N						AMINO ACID MINIMA [2]
	0	20	40	60	80	100	
Isoleucine	47.0	44.6	43.4	42.3	39.9	41.6	61.0
Leucine	80.0	80.5	81.3	79.4	72.0	74.0	72.5
Valine	54.8	56.0	54.5	46.4	45.8	43.8	51.0
Threonine	74.0	75.6	74.2	70.5	63.6	62.4	43.0
Methionine	27.3	27.8	27.6	26.2	23.9	23.4	39.0
Phenylalanine	48.2	44.4	45.0	45.0	42.0	39.6	45.0
Lysine	121.0	105.0	102.0	115.0	107.0	101.0	58.0
Histidine	27.2	26.3	26.8	27.4	22.9	23.1	21.5
Tryptophan [1]	16.8	15.9	14.9	13.2	8.1	2.8	14.5

[1] Colorimetric determination.

[2] Minimum daily requirements which support a rapid rate of recovery in protein-depleted rats (Cannon, '48; Frost, '50).

in protein-depleted rats (Cannon, '48; Frost, '50) are shown in the last column of the table. There was a progressive loss of amino acids, amounting generally to 10 to 20% after 100 minutes of autoclaving. The exception was tryptophan, in which case the loss after 100 minutes was more than 80% of the original amount.

Hydrolysis of the autoclaved, heat-darkened solutions for 8 hours at reflux temperature with 8 N hydrochloric acid liberated all of the amino acids, except tryptophan, as measured microbiologically. Table 3 shows amino acid concentrations

after acid hydrolysis of the same solutions described in table 2. After complete hydrolysis in strong acid there appears to be little difference in the amino acid composition of non-browned solutions and solutions which have undergone extensive browning. Methionine was partially destroyed by the acid, and tryptophan completely destroyed, as would be expected.

As indicated in figure 3, there was a progressive loss of nutritive value up to about 60 minutes of heating. This was

TABLE 3

Microbiological assay of heat-darkened fibrin hydrolysate-glucose solutions after acid hydrolysis

MINUTES AT 115°C.	MG AMINO ACID PER 200 MG TOTAL NITROGEN					
	0	20	40	60	80	100
Isoleucine	54.5	51.8	53.7	51.8	51.8	53.6
Leucine	94.2	97.2	93.8	96.0	94.0	93.4
Valine	67.2	68.4	67.5	67.0	67.3	68.6
Threonine	102.0	104.0	104.0	100.0	98.2	98.9
Methionine [1]	23.9	24.8	25.3	24.6	23.4	23.9
Phenylalanine	50.2	56.6	53.5	52.0	51.4	53.2
Lysine	117.0	112.0	115.0	110.0	104.0	119.0
Histidine	32.0	32.2	31.6	32.0	31.9	31.1

[1] Methionine was partially destroyed by acid hydrolysis. Tryptophan was completely destroyed.

followed by a very rapid nutritional deterioration. Intake of allotted nitrogen of the test hydrolysate was 100% throughout for the 20-, 40-, and 60-minute autoclaving samples. Intake decreased at 80 minutes to an average of 80% for 6 rats. At 100 minutes, the average intake fell to 43%. The analyses (table 2) indicated that the loss of tryptophan exceeded by far that of any other essential amino acid. At 60 minutes of heating the tryptophan level was 80% of the original amount, while after 80 minutes only 47% of the tryptophan remained.

An experimental fibrin hydrolysate with dextrose, initially low in tryptophan, gave improved repletion response on ad-

dition of tryptophan. On autoclaving the unfortified preparation 30 minutes at 115°C., the nutritive value dropped markedly while the tryptophan level fell only 15%. When tryptophan was added to this heat-darkened preparation, the rat repletion response observed was as great as that for the original hydrolysate. The level of tryptophan supplied by the original preparation was inadequate in proportion to the other essential amino acids. At the 200 mg N feeding level, it supplied only 13.8 mg tryptophan per rat per day, slightly below the minimum required for good response (table 2). The above-described extra autoclaving reduced this intake level only to 12.5 mg per day, but at the same time reduced the repletion response from an average of 9 to 2 gm for 6 rats for 5 days. When tryptophan was added to the autoclaved preparation, it supplied 17.9 mg tryptophan per rat per day, and the average response was 10.3 gm. This compares with an average improvement in repletion response from 9 to 15 gm on a similar addition of tryptophan to the original preparation. When the same amount of tryptophan was added originally and then autoclaved as above, the autoclaved preparation gave a 9.8 gm average response. Clearly, tryptophan was the most important limiting deficiency in this preparation throughout.

There was no significant heat loss of other essential amino acids as measured by microbiologcal assay of the completely hydrolyzed preparations. It is clear, nevertheless, from this and other studies, that long autoclaving makes other amino acids unavailable to the animal.

DISCUSSION

The relation between color and fluorescence development in progressively browned hydrolysate solutions suggests that the colored and fluorescing compounds are not identical. The fact that the peak of fluorescence precedes browning suggests that fluorogens may be precursors of the brown pigments. This idea is supported further by the effects of sulfite. in

which fluorogens accumulate and at the same time browning is inhibited. This result might be expected if sulfite inhibits a transformation of fluorogen to melanoidin, thus allowing fluorogen to accumulate. The measurement of fluorescence in very dark solutions is not quantitatively exact, in that with highly colored products there must be some absorption of the fluorescent energy. No study was made of the effects of dilution or concentration on fluorescence measurements. It is believed, however, that the results (fig. 2) represent the general trend in fluorescence, if not the absolute concentration of fluorogen. No doubt even in the absence of sulfite there was first a rapid build-up of fluorescing intermediates in the hydrolysate-glucose solutions before the first measurements were taken, as was the case with glycine-glucose solutions (table 1).

When heat-darkened hydrolysate-glucose solutions were injected subcutaneously in adult protein-depleted rats, highly colored products were excreted in the urine. On the other hand, there was no increased fluorescence in the urine corresponding to the high fluorescence of the injected material. Little is known about the metabolism of even primary condensation products of reducing sugars and amino acids, such as those described by Gottschalk and Partridge ('50).

The peak of fluorescence was well passed before any disturbance in nutritive value was noted either microbiologically or in rats. On the other hand, the development of color ran parallel both to the decrease in microbiological availability of many of the amino acids, and to the decrease in rat-repletion response. Thus, in hydrolysates originally well-balanced in amino acids, color change appears as a useful index for estimating changes in over-all nutritive value.[1]

In hydrolysates, the individual amino acids probably react with glucose at varying rates, depending on the chemical

[1] A plastic color standard, matching the solution color at which nutritive value begins to disappear rapidly, has been used satisfactorily for several years as an automatic dating device for a commercial protein hydrolysate solution with dextrose (Aminosol, 5% w/v with dextrose, U.S.P., 5% w/v).

and physical conditions, such as composition of the solution, concentration and temperature. The corresponding changes in over-all nutritive value are thus the results of many factors. Friedman and Kline ('50b) clearly indicated that the nutritive value was not adequately reflected by the amino acid analysis under their conditions of study. The same conclusion obtains in the present experiments. These studies suggest that tryptophan may become the most important limiting factor when it is originally present in marginal amounts. This is important in view of the fact that tryptophan is a crucially important amino acid, both biologically and economically. Often it may be present in hydrolysates in marginal amounts. Basu and Ray ('46) have suggested that the level of tryptophan in aged hydrolysate-glucose solutions be given primary consideration with regard to possible changes in nutritive value. This viewpoint receives strong support in the present studies.

SUMMARY

The effects of heat on the nutritive value of fibrin hydrolysate and dextrose solutions were studied. A significant decrease in rat repletion response was noted when: (1) the amino nitrogen had shown about a 6 to 8% decrease; (2) fluorescence had decreased to a low value following an early maximum; (3) color had developed to a marked degree following an initial induction period. Microbiological assay of the heat-darkened hydrolysate with dextrose indicated progressive loss of many of the individual essential amino acids. The greatest individual loss was in tryptophan. All amino acids except tryptophan were largely recoverable upon complete acid hydrolysis. Prolonged heating produced changes in the hydrolysate-glucose solutions which were not overcome by replacement of tryptophan alone. The amino acid composition of heat-damaged hydrolysate-glucose solutions determined by microbiological assay does not give a true index of nutritive value.

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INFLUENCE OF AGE AND OF SEX OF THE ALBINO RAT ON HEPATIC ASCORBIC ACID[1]

A. L. MOREHOUSE[2] AND N. B. GUERRANT
Department of Agricultural and Biological Chemistry, The Pennsylvania State College, State College

THREE FIGURES

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INTRODUCTION

A great many reports dealing with the ascorbic acid content of the tissues of the more common animals have appeared in scientific literature. The rat has received considerable attention in regard to studies of this type, largely because it was one of the first species shown to be capable of synthesizing ascorbic acid in excess of its own requirements.

Several investigators have reported that the concentration of ascorbic acid in certain tissues of the rat is higher in young animals than in mature rats (Kratinova and Bosis, '39; Giroud et al., '36; Mulinos et al., '42; Kratinov et al., '46). Brieger ('42) noted that in the newborn rat the hepatic concentration first increased, then gradually declined. Since the rat is capable of synthesizing ascorbic acid, the change in the ascorbic acid content of its tissues with age may not be the same as in other animals. However, Ried ('50), using guinea pigs, found that younger animals had greater capacity for the conservation of ingested ascorbic acid than older animals, an observation which suggests that the influence of age on tissue ascorbic acid is similar in both species.

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[2] Formerly Procter and Gamble Research Fellow, now associated with the Grain Processing Corporation, Muscatine, Iowa.

Todhunter and McMillan ('46) presented evidence to show that the ascorbic acid content of the blood of normal rats was considerably higher in males than in females. Additional evidence of sex differences in the ascorbic acid metabolism of the rat was presented by Sutton and co-workers ('42), who observed that the excretion of ascorbic acid by females was much lower than that by males.

In an earlier investigation in this laboratory one of us (M.) had occasion to determine the ascorbic acid content of the livers of rats of various ages ('50). In accordance with previous findings, the results indicated that younger animals had higher concentrations of ascorbic acid in their livers; furthermore, there was a suggestion of a sex difference in the hepatic concentration of this vitamin. In view of earlier observations of Todhunter and McMillan ('46) of a sex difference in the concentration of ascorbic acid in the blood, it appeared desirable to learn if the hepatic concentration of this vitamin in normal rats also differed with respect to sex. The present investigation was undertaken to learn more about the influence of age and of sex upon the hepatic ascorbic acid of normal, healthy rats.

EXPERIMENTAL

To eliminate the possible effects of strain and dietary differences on ascorbic acid metabolism, all animals used in this study were albino rats obtained directly from the breeding colony and all rats subsisted on the same diet (a commercial breeding ration). In order to study the change in hepatic ascorbic acid during the first three weeks of life, livers were obtained from fetal rats just prior to birth and from suckling animals of 7, 14 and 21 days of age. Older animals, from 4 to 22 weeks of age, were provided by selecting a group of weanling rats of both sexes and maintaining them on the breeding ration, fed ad libitum, for the desired period. All animals except the unweaned young and those over 22 weeks of age were weighed at weekly inter-

vals, and food consumptions were recorded for the same period. Beginning with 4-week-old rats and at various ages thereafter, 4 or more representative animals of each sex were sacrificed and the ascorbic acid content of their individual livers determined. All animals over 22 weeks of age were taken from the breeding colony and sacrificed immediately preliminary to the determination of hepatic ascorbic acid.

Early in the investigation it was noted that the hepatic ascorbic acid of adult female rats was considerably higher when the animal was pregnant. Further study of this observation was carried out by mating a number of females and determining the ascorbic acid content of their livers at various stages of pregnancy. Several females with suckling young were sacrificed in order to determine the hepatic content of ascorbic acid during the lactation period.

Several groups of young rats which had received an experimental type of diet (deficient in vitamin A) were assayed for hepatic ascorbic acid as part of the present investigation. It was believed this additional data might indicate whether sex differences in hepatic ascorbic acid were influenced by the composition of the diet.

Samples of blood plasma obtained from some of the rats used in the present studies were assayed for ascorbic acid in order to compare the content of ascorbic acid in the blood of male and female rats.

Ascorbic acid was determined on individual livers except in very young rats, where several livers were pooled to yield an adequate sample. The animal was sacrificed by decapitation and the entire liver dissected out, pressed between filter paper, and weighed. The whole liver was extracted with 6% metaphosphoric acid solution in a Waring Blendor and the extract diluted with an equal volume of water. Reduced ascorbic acid was determined by titrating an aliquot of the filtered extract with a standardized solution of 2,6-dichlorobenzenoneindophenol. Measurements of reduced plus dehydroascorbic acid were also made, using the 2,4-dinitrophenylhy-

drazine method of Roe and Osterling ('44). The suggestion of Bolin and Book ('47), that 2,6-dichlorobenzenoneindophenol be used in place of norite or bromine for oxidizing ascorbic acid, was followed since it permitted the use of the same acid extract as was used in the visual titration method.

RESULTS AND DISCUSSION

The phenylhydrazine method of determining ascorbic acid (reduced plus dehydro forms) usually yielded a higher value for hepatic ascorbic acid than did the dye titration method (table 1). This suggests that perhaps some dehydroascorbic acid is present in the liver of the rat. It is also probable that slight oxidation of the vitamin occurred during the process of extraction and filtration prior to the actual determination. However, when the ascorbic acid values obtained by the two methods of assay were compared, it was of interest to find that the livers of suckling rats yielded higher ascorbic acid values by the dye titration method. This would seem to indicate that these livers contained materials other than ascorbic acid which were capable of reducing solutions of 2,6-dichlorobenzenoneindophenol. However, the presence of such substances was not confirmed.

Influence of age on hepatic ascorbic acid

The data obtained in the present studies show that the concentration of ascorbic acid in the liver of the albino rat undergoes definite changes with age, particularly during the early postnatal life of the animal. The liver of the fetal rat just prior to birth contained about 0.22 mg of ascorbic acid per gram of tissue, a level which corresponded closely to the hepatic concentration of the vitamin observed for the mother rat. A marked, two-fold increase in hepatic ascorbic acid occurred during the first and second weeks of extrauterine life (fig. 1), the livers of two-week-old rats containing an average of 0.48 mg of ascorbic acid per gram of tissue. Since the concentration of ascorbic acid in the liver of

TABLE 1

Relation of age and sex of the rat to the concentration of ascorbic acid in the liver

AGE IN WEEKS	SEX	NO. OF ANIMALS	BY DYE TITRATION METHOD		BY PHENYLHYDRAZINE METHOD		RATIO: PHENYLHYDRAZINE METHOD
			Conc.	S.D. [1]	Conc.	S.D. [1]	Dye titration method
			mg/gm		*mg/gm*		
0 [2]	?	2	0.22	0.042	0.23	0.057	1.05
1	?	8	0.37	0.051	0.30	0.045	0.81
2	M	8	0.48	0.085	0.40	0.016	0.83
	F	8	0.47	0.010	0.40	0.021	0.85
3	M	6	0.50	0.032	0.43	0.031	0.86
	F	6	0.46	0.038	0.39	0.033	0.85
4	M	9	0.33	0.051	0.36	0.033	1.09
	F	9	0.28	0.035	0.30	0.027	1.07
6	M	8	0.26	0.060	0.27	0.057	1.04
	F	8	0.21	0.020	0.21	0.018	1.00
8	M	4	0.30	0.019	0.35	0.019	1.17
	F	4	0.19	0.032	0.23	0.039	1.21
10	M	4	0.29	0.039	0.32	0.022	1.10
	F	4	0.20	0.029	0.22	0.039	1.10
14	M	4	0.25	0.028	0.28	0.031	1.12
	F	4	0.19	0.014	0.21	0.017	1.11
18	M	4	0.27	0.050	0.29	0.039	1.07
	F	4	0.19	0.031	0.20	0.026	1.05
22	M	6	0.25	0.044	0.30	0.044	1.20
	F	4	0.18	0.033	0.21	0.044	1.17
34	M	5	0.28	0.027	0.30	0.032	1.07
	F	5	0.18	0.019	0.20	0.016	1.11
40	M	6	0.23	0.019			
	F	6	0.15	0.015			
42	M	6	0.24	0.017	0.27	0.020	1.12
	F	4	0.15	0.006	0.18	0.015	1.20

[1] Standard deviation.

[2] Fetal rats.

the three-week-old rat did not differ appreciably from that of the two-week-old animal, the concentration apparently reaches its highest level during the third week of life.

A rapid decrease in ascorbic acid concentration occurred during the 4th week of life, and this decrease was followed by further lowering of the vitamin concentration during the 5th and 6th weeks of postnatal life. Inasmuch as the rats

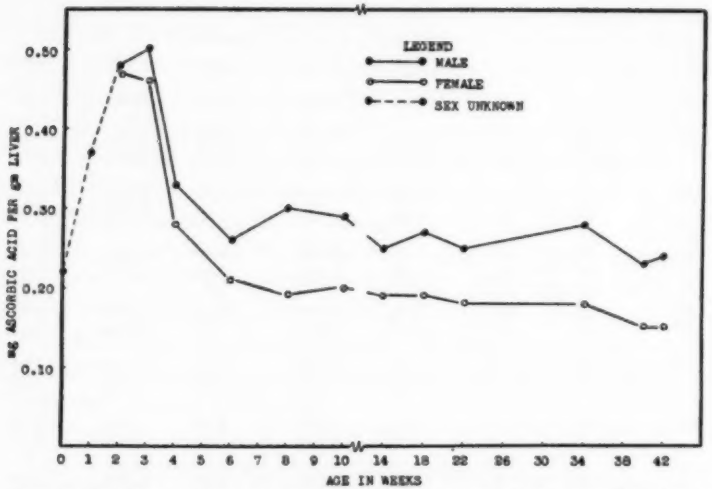

Fig. 1 Relation of age and sex of the albino rat to the concentration of ascorbic acid in the liver. Each point represents the mean of 4 to 9 individual determinations.

were weaned when they were between three and 4 weeks of age, the rapid decline in hepatic ascorbic acid which occurred at this time may have been associated with the removal of the dam's milk from the diet of the young rats.

The results of the present studies show that the hepatic ascorbic acid concentration in rats over 8 weeks of age remains relatively constant, although a measurable and gradual decrease in concentration was observed in the livers of both sexes up to 42 weeks of age (table 1, fig. 1).

Influence of sex on hepatic ascorbic acid

The curves presented in figure 1 show that the concentration of ascorbic acid in the livers of normal albino rats of corresponding age groups differs appreciably with respect to sex. In rats over two weeks of age the average hepatic concentration of ascorbic acid was always higher in the male than in the female of corresponding age, although from two to 4 weeks of age the difference in ascorbic acid concentrations between the sexes was not great. At above 6 weeks of age the difference between the concentration values for male and female rats seemed to increase and a distinct sex difference in the hepatic concentration of ascorbic acid became readily apparent. It was of interest to find that this increase in the difference in ascorbic acid concentration between sexes occurred at about the same time that the animals matured sexually.

The present data also indicate a greater uniformity of hepatic ascorbic acid values for different female rats than for different male rats. The two concentration curves presented in figure 1 illustrate this point. It will be noted that the curve for the female rat is relatively smooth, while the curve representing the hepatic ascorbic acid in male rats is quite irregular. The greater irregularity in the average concentration of ascorbic acid in the livers of male rats apparently reflects the greater individual variation between males as compared to females. An examination of the data presented in table 1 shows that for most of the age groups reported the standard deviation of the mean hepatic ascorbic acid concentration is larger for the male rats than for the corresponding female rats.

In view of the opinion expressed by Mapson and Walker ('48) that the concentration of ascorbic acid in the tissues of the rat is influenced by food intake, it seemed reasonable to consider whether or not the sex difference in hepatic ascorbic acid values observed in the present studies is attributable to differences in the amount of food consumed.

The data presented in figure 2 show that beyond 6 weeks of age the weekly food intake of male rats was considerably greater than that of female rats. As expected, male rats grew faster and a wide difference in the average body weight

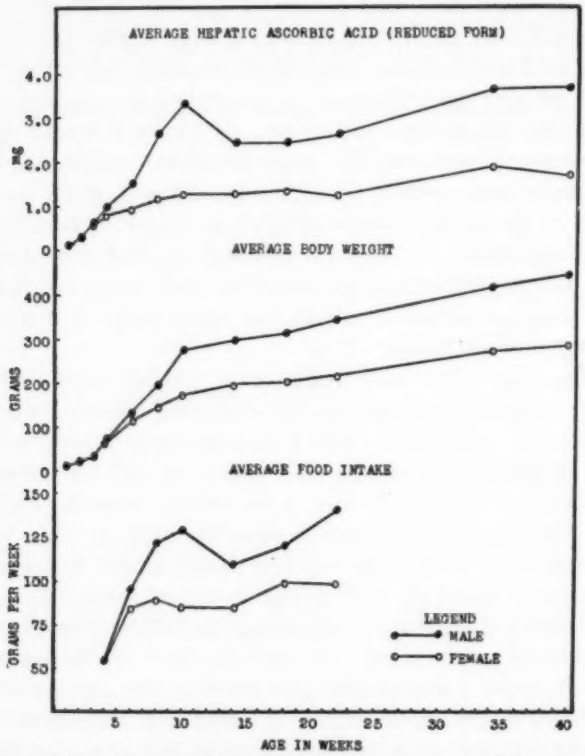

Fig. 2 Relation of age and sex of the albino rat to total hepatic ascorbic acid, body weight and food intake.

and liver weight of the two sexes was evident by the time the animals were 10 weeks old. The data presented do indicate some correlation between weekly food intakes and the total reduced ascorbic acid found in the livers of male and female rats. However, a consideration of individual ani-

mals failed to show that those animals consuming the most food regularly had the highest hepatic concentration of the vitamin. Furthermore, the data presented in table 2 indicate that the sex difference in hepatic ascorbic acid concentration certainly is not due entirely to differences in food intake. With both groups of rats (groups 1 and 2) the average food intake of the males was less than that of the corresponding females, yet with both groups the males were found to have higher hepatic concentrations of ascorbic acid. Thus, it ap-

TABLE 2

Relation of sex of rats maintained on the U.S.P. vitamin A-free diet plus various supplements to hepatic ascorbic acid

GROUP	TYPE OF SUPPLEMENT	SEX	NO. OF ANIMALS	AVE. FOOD INTAKE[1]	AVE. BODY WEIGHT	AVE. LIVER WEIGHT	ASCORBIC ACID	
							Ave. Total	Conc.
				gm	*gm*	*gm*	*mg*	*mg/gm*
1	1.5 units vitamin A acetate daily	M	5	254	178	6.81	1.97	0.29 ± 0.078
		F	4	272	170	5.72	1.48	0.26 ± 0.016
2	1.5 units vitamin A acetate + 1 mg tocopherol daily	M	5	234	167	6.40	2.02	0.32 ± 0.015
		F	6	270	170	5.34	1.24	0.23 ± 0.015

[1] Food consumptions are for a 28-day test period.

pears that the sex difference observed in these studies is a real one and is probably characteristic of the normal albino rat.

Occasional determinations of blood plasma ascorbic acid (reduced + dehydro forms) were carried out during the investigation, using a pooled sample of plasma collected from two or more rats of the same age and sex. In each instance the plasma of male rats was found to have a higher concentration of ascorbic acid than the plasma of female rats. In addition, evidence was obtained which indicates that blood

plasma ascorbic acid declines with increasing age. At 18 weeks of age the plasma of male and female rats contained, respectively, 0.68 and 0.40 mg of ascorbic acid per 100 ml of plasma. Thus the results obtained in the present studies confirm the findings of Todhunter and McMillan ('46) with respect to a sex difference in the ascorbic acid content of the blood plasma of rats. Furthermore, the occurrence of a sex difference in the ascorbic acid content of blood plasma lends further support to the observation made in the pres-

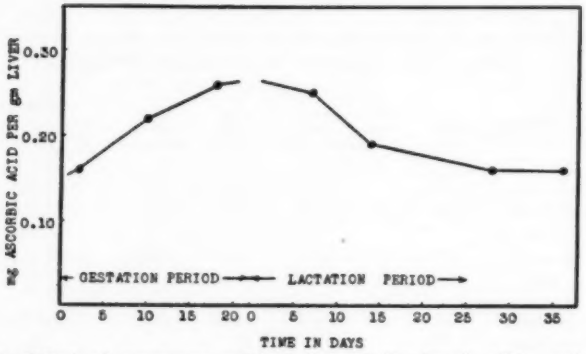

Fig. 3 Effect of pregnancy and lactation of the female albino rat on the concentration of ascorbic acid in the liver.

ent studies of a similar sex difference in the hepatic concentration of ascorbic acid.

Influence of pregnancy and lactation on hepatic ascorbic acid

When mature female rats were mated and then sacrificed at various stages of gestation, it was found that the concentration of ascorbic acid in the liver increased progressively as pregnancy advanced, the highest level being attained just before parturition (table 3, fig. 3). The increase in liver ascorbic acid during pregnancy as observed in this investigation agrees closely with the findings previously reported by

TABLE 3

Ascorbic acid content of livers of female rats sacrificed during pregnancy and lactation

ANIMAL NUMBER	STAGE OF GESTATION [1]	STAGE OF LACTATION	BODY WEIGHT	LIVER WEIGHT	TITRATION METHOD		PHENYLHYDRAZINE METHOD	
					Total	Conc.	Total	Conc.
	days	*days*	*gm*	*gm*	*mg*	*mg/gm*	*mg*	*mg/gm*
4733	2–3		286	10.10	1.48	0.15	1.60	0.16
4821	2–3		287	9.38	1.60	0.17	1.81	0.19
Ave.			287	9.74	1.54	0.16	1.71	0.18
4791	7–9		324	11.96	2.49	0.21	2.94	0.25
4781	10–12		283	11.83	2.43	0.20	2.80	0.24
4816	10–12		280	11.41	2.69	0.24	3.15	0.28
Ave.	10		296	11.73	2.54	0.22	2.96	0.26
4764	17–18		360	8.61	2.59	0.30	3.07	0.36
4767	17–18		367	10.14	2.71	0.27	3.02	0.30
4786	17–18		290	8.36	1.93	0.23	2.17	0.26
4774	19–20		357	9.75	2.18	0.22	2.63	0.27
Ave.	18		344	9.22	2.35	0.26	2.72	0.30
4766		7	274	9.48	2.13	0.23	2.23	0.24
4822		7	255	9.64	2.65	0.27	2.72	0.28
Ave.			265	9.56	2.39	0.25	2.48	0.26
4817		14	238	10.03	1.89	0.19	1.98	0.20
4780		14	274	13.56	2.58	0.19	2.61	0.19
Ave.			256	11.80	2.24	0.19	2.30	0.20
4768		28 [2]	290	14.00	1.90	0.14	2.39	0.17
4802		28 [2]	313	11.74	1.82	0.16	2.20	0.19
4824		28 [2]	348	10.91	1.83	0.17	2.10	0.19
Ave.			317	12.22	1.85	0.16	2.23	0.18
4769		[3]	243	10.07	1.73	0.17	2.01	0.20
4784		[3]	250	9.06	1.35	0.15	1.59	0.18
Ave.			247	9.57	1.54	0.16	1.80	0.19

[1] The stage of gestation was estimated from the appearance of the embryos.
[2] One to three days after weaning young.
[3] Eleven days after weaning young.

Kennaway and Tipler ('47). However, the present data show a greater increase in concentration during pregnancy than that reported by the aforementioned authors. In the present investigation the liver of the non-pregnant female rat contained 0.15 mg ascorbic acid per gram of liver, while the liver of the pregnant female, during the late stages of gestation, contained 0.26 mg of the vitamin per gram of tissue. This difference represents a 75% increase in concentration as a result of, or associated with, pregnancy.

It was of interest to observe that the decline in liver ascorbic acid of female rats following parturition was nearly the reverse of the increase which occurred during pregnancy. The data presented in figure 3 show that by the end of the lactation period the hepatic concentration of ascorbic acid had returned to the level found in the non-pregnant female rat. The concentration apparently remains at this level unless a subsequent pregnancy occurs.

SUMMARY

A study has been made of the influence of the age and of the sex of the albino rat on the hepatic content of ascorbic acid. These studies involved measurement of the ascorbic acid in the livers of male and female rats of various ages, ranging from suckling young to mature rats 42 weeks of age, while they were being maintained on a commercial breeding ration. The present investigation also includes a study of the effect of pregnancy and lactation on hepatic ascorbic acid. The results of these studies may be summarized as follows:

1. A two-fold increase in the hepatic concentration of ascorbic acid occurred during the first three weeks of extrauterine life. This early increase in ascorbic acid concentration was followed by a rapid decrease during the 4th week of life and may have been associated with the weaning of the rat.

2. The concentration of hepatic ascorbic acid in rats over 8 weeks of age remained relatively constant, although a meas-

urable gradual decrease in concentration of the vitamin was observed in the livers of both sexes up to 42 weeks of age.

3. With rats exceeding 6 weeks of age a distinct sex difference in the hepatic concentration of ascorbic acid was observed, the concentration of the vitamin being significantly higher in the livers of the males. Although food intakes usually were higher for male rats, no definite evidence was obtained to show that the sex difference in hepatic concentrations of the vitamin can be attributed to differences in food intake.

4. The occurrence of a sex difference in the ascorbic acid content of the blood plasma of rats was confirmed in the present studies.

5. The concentration of ascorbic acid in the livers of pregnant female rats was observed to increase progressively as pregnancy advanced, reaching a concentration just before parturition that was 75% higher than that of the non-pregnant female. The hepatic concentration of the vitamin decreased gradually following parturition, and by the end of the lactation period had returned to the normal level of the non-pregnant female.

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